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USSN 09/330,235

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NIZATION

## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

<b>(51) International Patent Classification <sup>6</sup> :</b> <b>C12N 15/53, 15/82, A01H 5/00</b>	<b>A2</b>	<b>(11) International Publication Number:</b> <b>WO 96/21022</b> <b>(43) International Publication Date:</b> 11 July 1996 (11.07.96)
<b>(21) International Application Number:</b> PCT/IB95/01167 <b>(22) International Filing Date:</b> 28 December 1995 (28.12.95)  <b>(30) Priority Data:</b> 08/366,779 30 December 1994 (30.12.94) US  <b>(71) Applicant:</b> RHONE-POULENC AGROCHIMIE (FR/FR); 14-20, rue Pierre-Baizet, F-69263 Lyon (FR).  <b>(72) Inventors:</b> THOMAS, Terry, L.; 3004 Normand, College Station, TX 77845 (US). REDDY, Avum, S.; 3902 E. 29th Street #G11, Bryan, TX 77802 (US). NUCCIO, Michael; P.O. Box 553, College Station, TX 77841 (US). NUNBERG, Andrew, N.; 2804 B. Sprucewood Street, Bryan, TX 77801 (US). FREYSSINET, Georges, L.; 21, rue de Nervieux, F-69450 Saint-Cyr-au-Mont-d'Or (FR).  <b>(74) Agent:</b> MITSCHERLICH & PARTNER; Sonnenstrasse 33, D-80331 München (DE).	<b>(81) Designated States:</b> AU, BR, CA, CN, JP, RO, RU, UA, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).  <b>Published</b> <i>Without international search report and to be republished upon receipt of that report.</i>	
<b>(54) Title:</b> PRODUCTION OF GAMMA LINOLENIC ACID BY A $\Delta 6$ -DESATURASE  <b>(57) Abstract</b>  Linoleic acid is converted into $\gamma$ -linolenic acid by the enzyme $\Delta 6$ -desaturase. The present invention is directed to isolated nucleic acids comprising the $\Delta 6$ -desaturase gene. More particularly, the isolated nucleic acid comprises the promoter, coding region and termination regions of the $\Delta 6$ -desaturase gene. The present invention provides recombinant constructions comprising the $\Delta 6$ -desaturase coding region in functional combination with heterologous regulatory sequences. The nucleic acids and recombinant constructions of the instant invention are useful in the production of GLA in transgenic organisms.		

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GA	Gabon			VN	Viet Nam

420

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GAT CCC AAG AAT AGA CCA GAG ATC TGG GGA CGA TAC GCT CTT ATC TTT  
Asp Pro Lys Asn Arg Pro Glu Ile Trp Gly Arg Tyr Ala Leu Ile Phe

480

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GGA TCC TTG ATC GCT TCC TAC TAC GCG CAG CTC TTT GTG CCT TTC GTT  
Gly Ser Leu Ile Ala Ser Tyr Tyr Ala Gln Leu Phe Val Pro Phe Val

GTC GAA CGC ACA TGG CTT CAG GTG GTG TTT GCA ATC ATC ATG GGA TTT  
Val Glu Arg Thr Trp Leu Gln Val Val Phe Ala Ile Ile Met Gly Phe

540

\*

GCG TGC GCA CAA GTC GGA CTC AAC CCT CTT CAT GAT GCG TCT CAC TTT  
Ala Cys Ala Gln Val Gly Leu Asn Pro Leu His Asp Ala Ser His Phe

600

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TCA GTG ACC CAC AAC CCC ACT GTC TGG AAG ATT CTG GGA GCC ACG CAC  
Ser Val Thr His Asn Pro Thr Val Trp Lys Ile Leu Gly Ala Thr His

660

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GAC TTT TTC AAC GGA GCA TCG TAC CTG GTG TGG ATG TAC CAA CAT ATG  
Asp Phe Phe Asn Gly Ala Ser Tyr Leu Val Trp Met Tyr Gln His Met

720

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CTC GGC CAT CAC CCC TAC ACC AAC ATT GCT GGA GCA GAT CCC GAC GTG  
Leu Gly His His Pro Tyr Thr Asn Ile Ala Gly Ala Asp Pro Asp Val

TCG ACG TCT GAG CCC GAT GTT CGT CGT ATC AAG CCC AAC CAA AAG TGG  
Ser Thr Ser Glu Pro Asp Val Arg Arg Ile Lys Pro Asn Gln Lys Trp

780

\*

TTT GTC AAC CAC ATC AAC CAG CAC ATG TTT GTT CCT TTC CTG TAC GGA  
Phe Val Asn His Ile Asn Gln His Met Phe Val Pro Phe Leu Tyr Gly

840

\*

CTG CTG GCG TTC AAG GTG CGC ATT CAG GAC ATC AAC ATT TTG TAC TTT  
Leu Leu Ala Phe Lys Val Arg Ile Gln Asp Ile Asn Ile Leu Tyr Phe

900

\*

GTC AAG ACC AAT GAC GCT ATT CGT GTC AAT CCC ATC TCG ACA TGG CAC  
Val Lys Thr Asn Asp Ala Ile Arg Val Asn Pro Ile Ser Thr Trp His

960

\*

ACT GTG ATG TTC TGG GGC GGC AAG GCT TTC TTT GTC TGG TAT CGC CTG  
Thr Val Met Phe Trp Gly Gly Lys Ala Phe phe Val Trp Tyr Arg Leu

ATT GTT CCC CTG CAG TAT CTG CCC CTG GGC AAG GTG CTG CTC TTG TTC  
Ile Val Pro Leu Gln Tyr Leu Pro Leu Gly Lys Val Leu Leu Leu Phe

1020

\*

ACG GTC GCG GAC ATG GTG TCG TCT TAC TGG CTG GCG CTG ACC TTC CAG  
Thr Val Ala Asp Met Val Ser Ser Tyr Trp Leu Ala Leu Thr Phe Gln

1080

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GCG AAC CAC GTT GTT GAG GAA GTT CAG TGG CCG TTG CCT GAC GAG AAC  
Ala Asn His Val Val Glu Glu Val Gln Trp Pro Leu Pro Asp Glu Asn

1140

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GGG ATC ATC CAA AAG GAC TGG GCA GCT ATG CAG GTC GAG ACT ACG CAG  
Gly Ile Ile Gln Lys Asp Trp Ala Ala Met Gln Val Glu Thr Thr Gln

1200

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GAT TAC GCA CAC GAT TCG CAC CTC TGG ACC AGC ATC ACT GGC AGC TTG  
Asp Tyr Ala His Asp Ser His Leu Trp Thr Ser Ile Thr Gly Ser Leu

AAC TAC CAG GCT GTG CAC CAT CTG TTC CCC AAC GTG TCG CAG CAC CAT  
Asn Tyr Gln Ala Val His His Leu Phe Pro Asn Val Ser Gln His His

1260

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TAT CCC GAT ATT CTG GCC ATC ATC AAG AAC ACC TGC AGC GAG TAC AAG  
Tyr Pro Asp Ile Leu Ala Ile Ile Lys Asn Thr Cys Ser Glu Tyr Lys

1320

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GTT CCA TAC CTT GTC AAG GAT ACG TTT TGG CAA GCA TTT GCT TCA CAT  
Val Pro Tyr Leu Val Lys Asp Thr Phe Trp Gln Ala Phe Ala Ser His

1380

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TTG GAG CAC TTG CGT GTT CTT GGA CTC CGT CCC AAG GAA GAG TAGA  
Leu Glu His Leu Arg Val Leu Gly Leu Arg Pro Lys Glu Glu

1440

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AGAAAAAAG CGCCGAATGA AGTATGCCC CCTTTTCTC CAAGAATGCC AAAAGGAGAT  
CAAGTGACA TTCTCTATGA AGA

GCTTCCTCCA GTTCATCCCTC CATTTGCGCA CCTGCATTCT TTACGACCGT TAAGCAAG

60

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ATG GGA ACG GAC CAA GGA AAA ACC TTC ACC TGG GAA GAG CTG GCG GCC  
Met Gly Thr Asp Gln Gly Lys Thr Phe Thr Trp Glu Glu Leu Ala Ala

120

\*

CAT AAC ACC AAG AAG GAC GAC CTA CTC TTG GCC ATC CGC GGC AGG GTG TAC  
His Asn Thr Lys Asp Asp Leu Leu Leu Ala Ile Arg Gly Arg Val Tyr

180

\*

GAT GTC ACA AAG TTC TTG AGC CGC CAT CCT GGT GGA GTG GAC ACT CTC  
Asp Val Thr Lys Phe Leu Ser Arg His Pro Gly Gly Val Asp Thr Leu

240

\*

CTG CTC GGA GCT GGC CGA GAT GTT ACT CCG GTC TTT GAG ATG TAT CAC  
Leu Leu Gly Ala Gly Arg Asp Val Thr Pro Val phe Glu Met Tyr His

GCG TTT GGG GCT GCA GAT GCC ATT ATG AAG AAG TAC TAT GTC GGT ACA  
Ala Phe Gly Ala Ala Asp Ala Ile Met Lys Lys Tyr Tyr Val Gly Thr

300

\*

CTG GTC TCG AAT GAG CTG CCC ATC TTC CCG GAG CCA ACG GTG TTC CAC  
Leu Val Ser Asn Glu Leu Pro Ile Phe Pro Glu Pro Thr Val Phe His

360

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AAA ACC ATC AAG ACG AGA GTC GAG GGC TAC TTT ACG GAT CGG AAC ATT  
Lys Thr Ile Lys Thr Arg Val Glu Gly Tyr Phe Thr Asp Arg Asn Ile

1 PRODUCTION OF GAMMA LINOLENIC ACID BY A  $\Delta 6$ -DESATURASE

Linoleic acid (18:2) (LA) is transformed into gamma linolenic acid (18:3) (GLA) by the enzyme  
5  $\Delta 6$ -desaturase. When this enzyme, or the nucleic acid encoding it, is transferred into LA-producing cells, GLA is produced. The present invention provides nucleic acids comprising the  $\Delta 6$ -desaturase gene. More specifically, the nucleic acids comprise the  
10 promoters, coding regions and termination regions of the  $\Delta 6$ -desaturase genes. The present invention is further directed to recombinant constructions comprising a  $\Delta 6$ -desaturase coding region in functional combination with heterologous regulatory sequences.  
15 The nucleic acids and recombinant constructions of the instant invention are useful in the production of GLA in transgenic organisms.

Unsaturated fatty acids such as linoleic ( $C_{18}\Delta^{9,12}$ ) and  $\alpha$ -linolenic ( $C_{18}\Delta^{9,12,15}$ ) acids are essential  
20 dietary constituents that cannot be synthesized by vertebrates since vertebrate cells can introduce double bonds at the  $\Delta^9$  position of fatty acids but cannot introduce additional double bonds between the  $\Delta^9$  double bond and the methyl-terminus of the fatty  
25 acid chain. Because they are precursors of other products, linoleic and  $\alpha$ -linolenic acids are essential fatty acids, and are usually obtained from plant sources. Linoleic acid can be converted by mammals into  $\gamma$ -linolenic acid (GLA,  $C_{18}\Delta^{6,9,12}$ ) which can in turn  
30 be converted to arachidonic acid (20:4), a critically

1 important fatty acid since it is an essential precursor of most prostaglandins.

The dietary provision of linoleic acid, by virtue of its resulting conversion to GLA and  
5 arachidonic acid, satisfies the dietary need for GLA and arachidonic acid. However, a relationship has been demonstrated between consumption of saturated fats and health risks such as hypercholesterolemia, atherosclerosis and other clinical disorders which  
10 correlate with susceptibility to coronary disease, while the consumption of unsaturated fats has been associated with decreased blood cholesterol concentration and reduced risk of atherosclerosis. The therapeutic benefits of dietary GLA may result  
15 from GLA being a precursor to arachidonic acid and thus subsequently contributing to prostaglandin synthesis. Accordingly, consumption of the more unsaturated GLA, rather than linoleic acid, has potential health benefits. However, GLA is not  
20 present in virtually any commercially grown crop plant.

Linoleic acid is converted into GLA by the enzyme  $\Delta 6$ -desaturase.  $\Delta 6$ -desaturase, an enzyme of more than 350 amino acids, has a membrane-bound domain  
25 and an active site for desaturation of fatty acids. When this enzyme is transferred into cells which endogenously produce linoleic acid but not GLA, GLA is produced. The present invention, by providing the gene encoding  $\Delta 6$ -desaturase, allows the production of  
30 transgenic organisms which contain functional  $\Delta 6$ -desaturase and which produce GLA. In addition to



1 allowing production of large amounts of GLA, the  
present invention provides new dietary sources of GLA.

The present invention is directed to  
isolated  $\Delta 6$ -desaturase genes. Specifically, the  
5 isolated genes comprises the  $\Delta 6$ -desaturase promoters,  
coding regions, and termination regions.

The present invention is further directed to  
expression vectors comprising the  $\Delta 6$ -desaturase  
promoter, coding region and termination region.

10 Yet another aspect of this invention is  
directed to expression vectors comprising a  $\Delta 6$ -  
desaturase coding region in functional combination  
with heterologous regulatory regions, i.e. elements  
not derived from the  $\Delta 6$ -desaturase gene.

15 Cells and organisms comprising the vectors  
of the present invention, and progeny of such  
organisms, are also provided by the present invention.

A further aspect of the present invention  
provides isolated bacterial  $\Delta 6$ -desaturase. An  
20 isolated plant  $\Delta 6$ -desaturase is also provided.

Yet another aspect of this invention  
provides a method for producing plants with increased  
gamma linolenic acid content.

A method for producing chilling tolerant  
25 plants is also provided by the present invention.

Fig. 1 depicts the hydropathy profiles of  
the deduced amino acid sequences of Synechocystis  $\Delta 6$ -  
desaturase (Panel A) and  $\Delta 12$ -desaturase (Panel B).  
Putative membrane spanning regions are indicated by  
30 solid bars. Hydrophobic index was calculated for a

1 window size of 19 amino acid residues [Kyte, et al.  
(1982) J. Molec. Biol. 157].

Fig. 2 provides gas liquid chromatography  
profiles of wild type (Panel A) and transgenic (Panel  
5 B) Anabaena.

Fig. 3 is a diagram of maps of cosmid cSy75,  
cSy13 and Csy7 with overlapping regions and subclones.  
The origins of subclones of Csy75, Csy75-3.5 and Csy7  
are indicated by the dashed diagonal lines.  
10 Restriction sites that have been inactivated are in  
parentheses.

Fig. 4 provides gas liquid chromatography  
profiles of wild type (Panel A) and transgenic (Panel  
B) tobacco.

15 Fig. 5A depicts the DNA sequence of a  $\Delta$ -6  
desaturase cDNA isolated from borage.

Fig. 5B depicts the protein sequence of the  
open reading frame in the isolated borage  $\Delta$ -6  
desaturase cDNA. Three amino acid motifs  
20 characteristic of desaturases are indicated and are,  
in order, lipid box, metal box 1, and metal box 2.

Fig. 6 is a dendrogram showing similarity of  
the borage  $\Delta$ 6-desaturase to other membrane-bound  
desaturases. The amino acid sequence of the borage  
25  $\Delta$ 6-desaturase was compared to other known desaturases  
using Gene Works (IntelliGenetics). Numerical values  
correlate to relative phylogenetic distances between  
subgroups compared.

Fig. 7 is a restriction map of 221. $\Delta$ 6.NOS  
30 and 121. $\Delta$ 6.NOS. In 221. $\Delta$ 6.NOS, the remaining portion

1 of the plasmid is pBI221 and in 121.Δ6.NOS, the  
remaining portion of the plasmid is pBI121.

Fig. 8 provides gas liquid chromatography  
profiles of mock transfected (Panel A) and 221.Δ6.NOS  
5 transfected (Panel B) carrot cells. The positions of  
18:2, 18:3 α, and 18:3 γ(GLA) are indicated.

Fig. 9 provides gas liquid chromatography  
profiles of an untransformed tobacco leaf (Panel A)  
and a tobacco leaf transformed with 121.Δ6.NOS. The  
10 positions of 18:2, 18:3 α, 18:3γ(GLA), and 18:4 are  
indicated.

Fig. 10 provides gas liquid chromatography  
profiles for untransformed tobacco seeds (Panel A) and  
seeds of tobacco transformed with 121.Δ6.NOS. The  
15 positions of 18:2, 18:3α and 18:3γ(GLA) are indicated.

The present invention provides isolated  
nucleic acids encoding Δ6-desaturase. To identify a  
nucleic acid encoding Δ6-desaturase, DNA is isolated  
from an organism which produces GLA. Said organism  
20 can be, for example, an animal cell, certain fungi  
(e.g. Mortierella), certain bacteria (e.g.  
Synechocystis) or certain plants (borage, Oenothera,  
currants). The isolation of genomic DNA can be  
accomplished by a variety of methods well-known to one  
25 of ordinary skill in the art, as exemplified by  
Sambrook et al. (1989) in Molecular Cloning: A  
Laboratory Manual, Cold Spring Harbor, NY. The  
isolated DNA is fragmented by physical methods or  
enzymatic digestion and cloned into an appropriate  
30 vector, e.g. a bacteriophage or cosmid vector, by any  
of a variety of well-known methods which can be found

1 in references such as Sambrook et al. (1989).  
Expression vectors containing the DNA of the present  
invention are specifically contemplated herein. DNA  
encoding  $\Delta 6$ -desaturase can be identified by gain of  
5 function analysis. The vector containing fragmented  
DNA is transferred, for example by infection,  
transconjugation, transfection, into a host organism  
that produces linoleic acid but not GLA. As used  
herein, "transformation" refers generally to the  
10 incorporation of foreign DNA into a host cell.  
Methods for introducing recombinant DNA into a host  
organism are known to one of ordinary skill in the art  
and can be found, for example, in Sambrook et al.  
(1989). Production of GLA by these organisms (i.e.,  
15 gain of function) is assayed, for example by gas  
chromatography or other methods known to the  
ordinarily skilled artisan. Organisms which are  
induced to produce GLA, i.e. have gained function by  
the introduction of the vector, are identified as  
20 expressing DNA encoding  $\Delta 6$ -desaturase, and said DNA is  
recovered from the organisms. The recovered DNA can  
again be fragmented, cloned with expression vectors,  
and functionally assessed by the above procedures to  
define with more particularity the DNA encoding  $\Delta 6$ -  
25 desaturase.

As an example of the present invention,  
random DNA is isolated from the cyanobacteria  
Synechocystis Pasteur Culture Collection (PCC) 6803,  
American Type Culture Collection (ATCC) 27184, cloned  
30 into a cosmid vector, and introduced by  
transconjugation into the GLA-deficient cyanobacterium

1 Anabaena strain PCC 7120, ATCC 27893. Production of GLA from Anabaena linoleic acid is monitored by gas chromatography and the corresponding DNA fragment is isolated.

5 The isolated DNA is sequenced by methods well-known to one of ordinary skill in the art as found, for example, in Sambrook et al. (1989).

In accordance with the present invention, DNA molecules comprising  $\Delta 6$ -desaturase genes have been  
10 isolated. More particularly, a 3.588 kilobase (kb) DNA comprising a  $\Delta 6$ -desaturase gene has been isolated from the cyanobacteria Synechocystis. The nucleotide sequence of the 3.588 kb DNA was determined and is shown in SEQ ID NO:1. Open reading frames defining  
15 potential coding regions are present from nucleotide 317 to 1507 and from nucleotide 2002 to 3081. To define the nucleotides responsible for encoding  $\Delta 6$ -desaturase, the 3.588 kb fragment that confers  $\Delta 6$ -desaturase activity is cleaved into two subfragments,  
20 each of which contains only one open reading frame. Fragment ORF1 contains nucleotides 1 through 1704, while fragment ORF2 contains nucleotides 1705 through 3588. Each fragment is subcloned in both forward and reverse orientations into a conjugal expression vector  
25 (AM542, Wolk et al. [1984] Proc. Natl. Acad. Sci. USA 81, 1561) that contains a cyanobacterial carboxylase promoter. The resulting constructs (i.e. ORF1(F), ORF1(R), ORF2(F) and ORF2(R)) are conjugated to wild-type Anabaena PCC 7120 by standard methods (see, for  
30 example, Wolk et al. (1984) Proc. Natl. Acad. Sci. USA 81, 1561). Conjugated cells of Anabaena are

1 identified as Neo<sup>r</sup> green colonies on a brown  
background of dying non-conjugated cells after two  
weeks of growth on selective media (standard mineral  
media BG11N + containing 30µg/ml of neomycin according  
5 to Rippka et al., (1979) J. Gen Microbiol. 111, 1).  
The green colonies are selected and grown in selective  
liquid media (BG11N + with 15µg/ml neomycin). Lipids  
are extracted by standard methods (e.g. Dahmer et al.,  
(1989) Journal of American Oil Chemical Society 66,  
10 543) from the resulting transconjugants containing the  
forward and reverse oriented ORF1 and ORF2 constructs.  
For comparison, lipids are also extracted from wild-  
type cultures of Anabaena and Synechocystis. The  
fatty acid methyl esters are analyzed by gas liquid  
15 chromatography (GLC), for example with a Tracor-560  
gas liquid chromatograph equipped with a hydrogen  
flame ionization detector and a capillary column. The  
results of GLC analysis are shown in Table 1.

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1 Table 1: Occurrence of C18 fatty acids in wild-type  
and transgenic cyanobacteria

	SOURCE	18:0	18:1	18:2	$\gamma$ 18:3	$\alpha$ 18:3	18:4
5	Anabaena (wild type)	+	+	+	-	+	-
	Anabaena + ORF1 (F)	+	+	+	-	+	-
	Anabaena + ORF1 (R)	+	+	+	-	+	-
	Anabaena + ORF2 (F)	+	+	+	+	+	+
10	Anabaena + ORF2 (R)	+	+	+	-	+	-
	Synechocystis (wild type)	+	+	+	+	-	-

As assessed by GLC analysis, GLA deficient  
 15 Anabaena gain the function of GLA production when the  
 construct containing ORF2 in forward orientation is  
 introduced by transconjugation. Transconjugants  
 containing constructs with ORF2 in reverse orientation  
 to the carboxylase promoter, or ORF1 in either  
 20 orientation, show no GLA production. This analysis  
 demonstrates that the single open reading frame (ORF2)  
 within the 1884 bp fragment encodes  $\Delta$ 6-desaturase.  
 The 1884 bp fragment is shown as SEQ ID NO:3. This is  
 substantiated by the overall similarity of the  
 25 hydropathy profiles between  $\Delta$ 6-desaturase and  $\Delta$ 12-  
 desaturase [Wada et al. (1990) Nature 347] as shown in  
 Fig. 1 as (A) and (B), respectively.

Also in accordance with the present  
 invention, a cDNA comprising a  $\Delta$ 6-desaturase gene from  
 30 borage (Borago officinalis) has been isolated. The  
 nucleotide sequence of the 1.685 kilobase (kb) cDNA

1 was determined and is shown in Fig. 5A (SEQ ID NO: 4).  
The ATG start codon and stop codon are underlined.  
The amino acid sequence corresponding to the open  
reading frame in the borage delta 6-desaturase is  
5 shown in Fig. 5B (SEQ ID NO: 5).

Isolated nucleic acids encoding  $\Delta 6$ -  
desaturase can be identified from other GLA-producing  
organisms by the gain of function analysis described  
above, or by nucleic acid hybridization techniques  
10 using the isolated nucleic acid which encodes  
Synechocystis or borage  $\Delta 6$ -desaturase as a  
hybridization probe. Both genomic and cDNA cloning  
methods are known to the skilled artisan and are  
contemplated by the present invention. The  
15 hybridization probe can comprise the entire DNA  
sequence disclosed as SEQ. ID NO:1 or SEQ. ID NO:4, or  
a restriction fragment or other DNA fragment thereof,  
including an oligonucleotide probe. Methods for  
cloning homologous genes by cross-hybridization are  
20 known to the ordinarily skilled artisan and can be  
found, for example, in Sambrook (1989) and Beltz et  
al. (1983) Methods in Enzymology 100, 266.

In another method of identifying a delta 6-  
desaturase gene from an organism producing GLA, a cDNA  
25 library is made from poly-A<sup>+</sup> RNA isolated from  
polysomal RNA. In order to eliminate hyper-abundant  
expressed genes from the cDNA population, cDNAs or  
fragments thereof corresponding to hyper-abundant  
cDNAs genes are used as hybridization probes to the  
30 cDNA library. Non hybridizing plaques are excised and  
the resulting bacterial colonies are used to inoculate



1 liquid cultures and sequenced. For example, as a  
means of eliminating other seed storage protein cDNAs  
from a cDNA library made from borage polysomal RNA,  
cDNAs corresponding to abundantly expressed seed  
5 storage proteins are first hybridized to the cDNA  
library. The "subtracted" DNA library is then used to  
generate expressed sequence tags (ESTs) and such tags  
are used to scan a data base such as GenBank to  
identify potential desaturates.

10 Transgenic organisms which gain the function  
of GLA production by introduction of DNA encoding  $\Delta$ -  
desaturase also gain the function of  
octadecatetraeonic acid ( $18:4^{6,9,12,15}$ ) production.  
Octadecatetraeonic acid is present normally in fish  
15 oils and in some plant species of the Boraginaceae  
family (Craig et al. [1964] J. Amer. Oil Chem. Soc.  
41, 209-211; Gross et al. [1976] Can. J. Plant Sci.  
56, 659-664). In the transgenic organisms of the  
present invention, octadecatetraeonic acid results  
20 from further desaturation of  $\alpha$ -linolenic acid by  $\Delta 6$ -  
desaturase or desaturation of GLA by  $\Delta 15$ -desaturase.

The 359 amino acids encoded by ORF2, i.e.  
the open reading frame encoding Synechocystis  $\Delta 6$ -  
desaturase, are shown as SEQ. ID NO:2. The open  
25 reading frame encoding the borage  $\Delta 6$ -desaturase is  
shown in SEQ ID NO: 5. The present invention further  
contemplates other nucleotide sequences which encode  
the amino acids of SEQ ID NO:2 and SEQ ID NO: 5. It  
is within the ken of the ordinarily skilled artisan to  
30 identify such sequences which result, for example,  
from the degeneracy of the genetic code. Furthermore,

1 one of ordinary skill in the art can determine, by the  
gain of function analysis described hereinabove,  
smaller subfragments of the fragments containing the  
open reading frames which encode  $\Delta 6$ -desaturases.

5 The present invention contemplates any such  
polypeptide fragment of  $\Delta 6$ -desaturase and the nucleic  
acids therefor which retain activity for converting LA  
to GLA.

In another aspect of the present invention,  
10 a vector containing a nucleic acid of the present  
invention or a smaller fragment containing the  
promoter, coding sequence and termination region of a  
 $\Delta 6$ -desaturase gene is transferred into an organism,  
for example, cyanobacteria, in which the  $\Delta 6$ -desaturase  
15 promoter and termination regions are functional.  
Accordingly, organisms producing recombinant  $\Delta 6$ -  
desaturase are provided by this invention. Yet  
another aspect of this invention provides isolated  $\Delta 6$ -  
desaturase, which can be purified from the recombinant  
20 organisms by standard methods of protein purification.  
(For example, see Ausubel et al. [1987] Current  
Protocols in Molecular Biology, Green Publishing  
Associates, New York).

Vectors containing DNA encoding  $\Delta 6$ -  
25 desaturase are also provided by the present invention.  
It will be apparent to one of ordinary skill in the  
art that appropriate vectors can be constructed to  
direct the expression of the  $\Delta 6$ -desaturase coding  
sequence in a variety of organisms. Replicable  
expression vectors are particularly preferred.  
30 Replicable expression vectors as described herein are

1 DNA or RNA molecules engineered for controlled  
expression of a desired gene, i.e. the  $\Delta 6$ -desaturase  
gene. Preferably the vectors are plasmids,  
bacteriophages, cosmids or viruses. Shuttle vectors,  
5 e.g. as described by Wolk et al. (1984) Proc. Natl.  
Acad. Sci. USA, 1561-1565 and Bustos et al. (1991) J.  
Bacteriol. 174, 7525-7533, are also contemplated in  
accordance with the present invention. Sambrook et  
al. (1989), Goeddel, ed. (1990) Methods in Enzymology  
10 185 Academic Press, and Perbal (1988) A Practical  
Guide to Molecular Cloning, John Wiley and Sons, Inc.,  
provide detailed reviews of vectors into which a  
nucleic acid encoding the present  $\Delta 6$ -desaturase can be  
inserted and expressed. Such vectors also contain  
15 nucleic acid sequences which can effect expression of  
nucleic acids encoding  $\Delta 6$ -desaturase. Sequence  
elements capable of effecting expression of a gene  
product include promoters, enhancer elements, upstream  
activating sequences, transcription termination  
20 signals and polyadenylation sites. Both constitutive  
and tissue specific promoters are contemplated. For  
transformation of plant cells, the cauliflower mosaic  
virus (CaMV) 35S promoter and promoters which are  
regulated during plant seed maturation are of  
25 particular interest. All such promoter and  
transcriptional regulatory elements, singly or in  
combination, are contemplated for use in the present  
replicable expression vectors and are known to one of  
ordinary skill in the art. The CaMV 35S promoter is  
30 described, for example, by Restrepo et al. (1990)

1 Plant Cell 2, 987. Genetically engineered and mutated regulatory sequences are also contemplated.

The ordinarily skilled artisan can determine vectors and regulatory elements suitable for  
5 expression in a particular host cell. For example, a vector comprising the promoter from the gene encoding the carboxylase of Anabaena operably linked to the coding region of  $\Delta 6$ -desaturase and further operably linked to a termination signal from Synechocystis is  
10 appropriate for expression of  $\Delta 6$ -desaturase in cyanobacteria. "Operably linked" in this context means that the promoter and terminator sequences effectively function to regulate transcription. As a further example, a vector appropriate for expression  
15 of  $\Delta 6$ -desaturase in transgenic plants can comprise a seed-specific promoter sequence derived from helianthinin, napin, or glycinin operably linked to the  $\Delta 6$ -desaturase coding region and further operably linked to a seed termination signal or the nopaline  
20 synthase termination signal. As a still further example, a vector for use in expression of  $\Delta 6$ -desaturase in plants can comprise a constitutive promoter or a tissue specific promoter operably linked to the  $\Delta 6$ -desaturase coding region and further  
25 operably linked to a constitutive or tissue specific terminator or the nopaline synthase termination signal.

In particular, the helianthinin regulatory elements disclosed in applicant's copending U.S.  
30 Application Serial No. 682,354, filed April 8, 1991 and incorporated herein by reference, are contemplated

1 as promoter elements to direct the expression of the  
Δ6-desaturase of the present invention.

Modifications of the nucleotide sequences or  
regulatory elements disclosed herein which maintain  
5 the functions contemplated herein are within the scope  
of this invention. Such modifications include  
insertions, substitutions and deletions, and  
specifically substitutions which reflect the  
degeneracy of the genetic code.

10 Standard techniques for the construction of  
such hybrid vectors are well-known to those of  
ordinary skill in the art and can be found in  
references such as Sambrook et al. (1989), or any of  
the myriad of laboratory manuals on recombinant DNA  
15 technology that are widely available. A variety of  
strategies are available for ligating fragments of  
DNA, the choice of which depends on the nature of the  
termini of the DNA fragments. It is further  
contemplated in accordance with the present invention  
20 to include in the hybrid vectors other nucleotide  
sequence elements which facilitate cloning, expression  
or processing, for example sequences encoding signal  
peptides, a sequence encoding KDEL, which is required  
for retention of proteins in the endoplasmic reticulum  
25 or sequences encoding transit peptides which direct  
Δ6-desaturase to the chloroplast. Such sequences are  
known to one of ordinary skill in the art. An  
optimized transit peptide is described, for example,  
by Van den Broeck et al. (1985) Nature 313, 358.  
30 Prokaryotic and eukaryotic signal sequences are

1 disclosed, for example, by Michaelis et al. (1982)  
Ann. Rev. Microbiol. 36, 425.

A further aspect of the instant invention provides organisms other than cyanobacteria or plants  
5 which contain the DNA encoding the  $\Delta 6$ -desaturase of the present invention. The transgenic organisms contemplated in accordance with the present invention include bacteria, cyanobacteria, fungi, and plants and animals. The isolated DNA of the present invention  
10 can be introduced into the host by methods known in the art, for example infection, transfection, transformation or transconjugation. Techniques for transferring the DNA of the present invention into such organisms are widely known and provided in  
15 references such as Sambrook et al. (1989).

• A variety of plant transformation methods are known. The  $\Delta 6$ -desaturase gene can be introduced into plants by a leaf disk transformation-regeneration procedure as described by Horsch et al. (1985) Science  
20 227, 1229. Other methods of transformation, such as protoplast culture (Horsch et al. (1984) Science 223, 496; DeBlock et al. (1984) EMBO J. 2, 2143; Barton et al. (1983) Cell 32, 1033) can also be used and are within the scope of this invention. In a preferred  
25 embodiment plants are transformed with Agrobacterium-derived vectors. However, other methods are available to insert the  $\Delta 6$ -desaturase genes of the present invention into plant cells. Such alternative methods include biolistic approaches (Klein et al. (1987)  
30 Nature 327, 70), electroporation, chemically-induced DNA uptake, and use of viruses or pollen as vectors.

1           When necessary for the transformation  
method, the  $\Delta 6$ -desaturase genes of the present  
invention can be inserted into a plant transformation  
vector, e.g. the binary vector described by Bevan  
5 (1984) Nucleic Acids Res. 12, 8111. Plant  
transformation vectors can be derived by modifying the  
natural gene transfer system of Agrobacterium  
tumefaciens. The natural system comprises large Ti  
(tumor-inducing)-plasmids containing a large segment,  
10 known as T-DNA, which is transferred to transformed  
plants. Another segment of the Ti plasmid, the vir  
region, is responsible for T-DNA transfer. The T-DNA  
region is bordered by terminal repeats. In the  
modified binary vectors the tumor-inducing genes have  
15 been deleted and the functions of the vir region are  
utilized to transfer foreign DNA bordered by the T-DNA  
border sequences. The T-region also contains a  
selectable marker for antibiotic resistance, and a  
multiple cloning site for inserting sequences for  
20 transfer. Such engineered strains are known as  
"disarmed" A. tumefaciens strains, and allow the  
efficient transformation of sequences bordered by the  
T-region into the nuclear genomes of plants.

Surface-sterilized leaf disks are inoculated  
25 with the "disarmed" foreign DNA-containing A.  
tumefaciens, cultured for two days, and then  
transferred to antibiotic-containing medium.  
Transformed shoots are selected after rooting in  
medium containing the appropriate antibiotic,  
30 transferred to soil and regenerated.

1           Another aspect of the present invention  
provides transgenic plants or progeny of these plants  
containing the isolated DNA of the invention. Both  
monocotyledenous and dicotyledenous plants are  
5 contemplated. Plant cells are transformed with the  
isolated DNA encoding  $\Delta 6$ -desaturase by any of the  
plant transformation methods described above. The  
transformed plant cell, usually in a callus culture or  
leaf disk, is regenerated into a complete transgenic  
10 plant by methods well-known to one of ordinary skill  
in the art (e.g. Horsch et al. (1985) Science 227,  
1129). In a preferred embodiment, the transgenic  
plant is sunflower, oil seed rape, maize, tobacco,  
peanut or soybean. Since progeny of transformed  
15 plants inherit the DNA encoding  $\Delta 6$ -desaturase, seeds  
or cuttings from transformed plants are used to  
maintain the transgenic plant line.

The present invention further provides a  
method for providing transgenic plants with an  
20 increased content of GLA. This method includes  
introducing DNA encoding  $\Delta 6$ -desaturase into plant  
cells which lack or have low levels of GLA but contain  
LA, and regenerating plants with increased GLA content  
from the transgenic cells. In particular,  
25 commercially grown crop plants are contemplated as the  
transgenic organism, including, but not limited to,  
sunflower, soybean, oil seed rape, maize, peanut and  
tobacco.

The present invention further provides a  
30 method for providing transgenic organisms which  
contain GLA. This method comprises introducing DNA



1 encoding  $\Delta 6$ -desaturase into an organism which lacks or  
has low levels of GLA, but contains LA. In another  
embodiment, the method comprises introducing one or  
more expression vectors which comprise DNA encoding  
5  $\Delta 12$ -desaturase and  $\Delta 6$ -desaturase into organisms which  
are deficient in both GLA and LA. Accordingly,  
organisms deficient in both LA and GLA are induced to  
produce LA by the expression of  $\Delta 12$ -desaturase, and  
GLA is then generated due to the expression of  $\Delta 6$ -  
10 desaturase. Expression vectors comprising DNA  
encoding  $\Delta 12$ -desaturase, or  $\Delta 12$ -desaturase and  $\Delta 6$ -  
desaturase, can be constructed by methods of  
recombinant technology known to one of ordinary skill  
in the art (Sambrook *et al.*, 1989) and the published  
15 sequence of  $\Delta 12$ -desaturase (Wada *et al* [1990] Nature  
(London) 347, 200-203. In addition, it has been  
discovered in accordance with the present invention  
that nucleotides 2002-3081 of SEQ. ID NO:1 encode  
cyanobacterial  $\Delta 12$ -desaturase. Accordingly, this  
20 sequence can be used to construct the subject  
expression vectors. In particular, commercially grown  
crop plants are contemplated as the transgenic  
organism, including, but not limited to, sunflower,  
soybean, oil seed rape, maize, peanut and tobacco.

25 The present invention is further directed to  
a method of inducing chilling tolerance in plants.  
Chilling sensitivity may be due to phase transition of  
lipids in cell membranes. Phase transition  
temperature depends upon the degree of unsaturation of  
fatty acids in membrane lipids, and thus increasing  
30 the degree of unsaturation, for example by introducing

1  $\Delta 6$ -desaturase to convert LA to GLA, can induce or  
improve chilling resistance. Accordingly, the present  
method comprises introducing DNA encoding  $\Delta 6$ -  
desaturase into a plant cell, and regenerating a plant  
5 with improved chilling resistance from said  
transformed plant cell. In a preferred embodiment,  
the plant is a sunflower, soybean, oil seed rape,  
maize, peanut or tobacco plant.

The following examples further illustrate  
10 the present invention.

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## EXAMPLE 1

## Strains and Culture Conditions

- Synechocystis (PCC 6803, ATCC 27184),  
5 Anabaena (PCC 7120, ATCC 27893) and Synechococcus (PCC  
7942, ATCC 33912) were grown photoautotrophically at  
30°C in BG11N+ medium (Rippka et al. [1979] J. Gen.  
Microbiol. 111, 1-61) under illumination of  
incandescent lamps  
10 ( $60\mu\text{E}\cdot\text{m}^{-2}\cdot\text{S}^{-1}$ ). Cosmids and plasmids were selected and  
propagated in Escherichia coli strain DH5 $\alpha$  on LB  
medium supplemented with antibiotics at standard  
concentrations as described by Maniatis et al. (1982)  
Molecular Cloning: A Laboratory Manual, Cold Spring  
15 Harbor Laboratory, Cold Spring, New York.

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## EXAMPLE 2

Construction of Synechocystis Cosmid Genomic Library

Total genomic DNA from Synechocystis (PCC  
5 6803) was partially digested with Sau3A and  
fractionated on a sucrose gradient (Ausubel et al.  
[1987] Current Protocols in Molecular Biology, Greene  
Publishing Associates and Wiley Interscience, New  
York). Fractions containing 30 to 40 kb DNA fragments  
10 were selected and ligated into the dephosphorylated  
BamHI site of the cosmid vector, pDUCA7 (Buikema et  
al. [1991] J. Bacteriol. 173, 1879-1885). The ligated  
DNA was packaged in vitro as described by Ausubel et  
al. (1987), and packaged phage were propagated in E.  
15 coli DH5 $\alpha$  containing the AvaI and Eco4711 methylase  
helper plasmid, pRL528 as described by Buikema et al.  
(1991). A total of 1152 colonies were isolated  
randomly and maintained individually in twelve 96-well  
microtiter plates.

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## EXAMPLE 3

Gain-of-Function Expression of GLA in Anabaena

Anabaena (PCC 7120), a filamentous cyanobacterium, is deficient in GLA but contains significant amounts of linoleic acid, the precursor for GLA (Figure 2; Table 2). The Synechocystis cosmid library described in Example 2 was conjugated into Anabaena (PCC 7120) to identify transconjugants that produce GLA. Anabaena cells were grown to mid-log phase in BG11N+ liquid medium and resuspended in the same medium to a final concentration of approximately  $2 \times 10^8$  cells per ml. A mid-log phase culture of E. coli RP4 (Burkardt et al. [1979] J. Gen. Microbiol. 114, 341-348) grown in LB containing ampicillin was washed and resuspended in fresh LB medium. Anabaena and RP4 were then mixed and spread evenly on BG11N+ plates containing 5% LB. The cosmid genomic library was replica plated onto LB plates containing 50  $\mu\text{g/ml}$  kanamycin and 17.5  $\mu\text{g/ml}$  chloramphenicol and was subsequently patched onto BG11N+ plates containing Anabaena and RP4. After 24 hours of incubation at 30°C, 30  $\mu\text{g/ml}$  of neomycin was underlaid; and incubation at 30°C was continued until transconjugants appeared.

Individual transconjugants were isolated after conjugation and grown in 2 ml BG11N+ liquid medium with 15  $\mu\text{g/ml}$  neomycin. Fatty acid methyl esters were prepared from wild type cultures and cultures containing pools of ten transconjugants as follows. Wild type and transgenic cyanobacterial

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1 cultures were harvested by centrifugation and washed  
twice with distilled water. Fatty acid methyl esters  
were extracted from these cultures as described by  
Dahmer et al. (1989) J. Amer. Oil. Chem. Soc. 66, 543-  
5 548 and were analyzed by Gas Liquid Chromatography  
(GLC) using a Tracor-560 equipped with a hydrogen  
flame ionization detector and capillary column (30 m x  
0.25 mm bonded FSOT Superox II, Alltech Associates  
Inc., IL). Retention times and co-chromatography of  
10 standards (obtained from Sigma Chemical Co.) were used  
for identification of fatty acids. The average fatty  
acid composition was determined as the ratio of peak  
area of each C18 fatty acid normalized to an internal  
standard.

15 • Representative GLC profiles are shown in  
Fig. 2. C18 fatty acid methyl esters are shown.  
Peaks were identified by comparing the elution times  
with known standards of fatty acid methyl esters and  
were confirmed by gas chromatography-mass  
20 spectrometry. Panel A depicts GLC analysis of fatty  
acids of wild type Anabaena. The arrow indicates the  
migration time of GLA. Panel B is a GLC profile of  
fatty acids of transconjugants of Anabaena with  
pAM542+1.8F. Two GLA producing pools (of 25 pools  
25 representing 250 transconjugants) were identified that  
produced GLA. Individual transconjugants of each GLA  
positive pool were analyzed for GLA production; two  
independent transconjugants, AS13 and AS75, one from  
each pool, were identified which expressed significant  
30 levels of GLA and which contained cosmids, cSy13 and  
cSy75, respectively (Figure 3). The cosmids overlap

1 in a region approximately 7.5 kb in length. A 3.5 kb  
NheI fragment of cSy75 was recloned in the vector  
pDUCA7 and transferred to Anabaena resulting in gain-  
of-function expression of GLA (Table 2).

5 Two NheI/Hind III subfragments (1.8 and 1.7  
kb) of the 3.5 kb Nhe I fragment of cSy75-3.5 were  
subcloned into "pBLUESCRIPT" (Stratagene) (Figure 3)  
for sequencing. Standard molecular biology techniques  
were performed as described by Maniatis et al. (1982)  
10 and Ausubel et al. (1987). Dideoxy sequencing (Sanger  
et al. [1977] Proc. Natl. Acad. Sci. USA 74, 5463-  
5467) of pBS1.8 was performed with "SEQUENASE" (United  
States Biochemical) on both strands by using specific  
oligonucleotide primers synthesized by the Advanced  
15 DNA Technologies Laboratory (Biology Department, Texas  
A & M University). DNA sequence analysis was done  
with the GCG (Madison, WI) software as described by  
Devereux et al. (1984) Nucleic Acids Res. 12, 387-395.

Both NheI/HindIII subfragments were  
20 transferred into a conjugal expression vector, AM542,  
in both forward and reverse orientations with respect  
to a cyanobacterial carboxylase promoter and were  
introduced into Anabaena by conjugation.  
Transconjugants containing the 1.8 kb fragment in the  
25 forward orientation (AM542-1.8F) produced significant  
quantities of GLA and octadecatetraenoic acid (Figure  
2; Table 2). Transconjugants containing other  
constructs, either reverse oriented 1.8 kb fragment or  
forward and reverse oriented 1.7 kb fragment, did not  
30 produce detectable levels of GLA (Table 2).

1           Figure 2 compares the C18 fatty acid profile  
of an extract from wild type Anabaena (Figure 2A) with  
that of transgenic Anabaena containing the 1.8 kb  
fragment of cSy75-3.5 in the forward orientation  
5 (Figure 2B). GLC analysis of fatty acid methyl esters  
from AM542-1.8F revealed a peak with a retention time  
identical to that of authentic GLA standard. Analysis  
of this peak by gas chromatography-mass spectrometry  
(GC-MS) confirmed that it had the same mass  
10 fragmentation pattern as a GLA reference sample.  
Transgenic Anabaena with altered levels of  
polyunsaturated fatty acids were similar to wild type  
in growth rate and morphology.

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1 Table 2 Composition of C18 Fatty Acids in Wild Type  
and Transgenic Cyanobacteria

5	Strain	Fatty Acid (%)					
		18:0	18:1	18:2	18:3( $\alpha$ )	18:3( $\gamma$ )	18:4
	<b>Wild Type</b>						
10	<i>Synechocystis</i> (sp.PCC6803)	13.6	4.5	54.5	-	27.3	-
	<i>Anabaena</i> (sp.PCC7120)	2.9	24.8	37.1	35.2	-	-
15	<i>Synechococcus</i> (sp.PCC7942)	20.6	79.4	-	-	-	-
	<b>Anabaena Transconjugants</b>						
	cSy75	3.8	24.4	22.3	9.1	27.9	12.5
	cSy75-3.5	4.3	27.6	18.1	3.2	40.4	6.4
20	pAM542 - 1.8P	4.2	13.9	12.1	19.1	25.4	25.4
	pAM542 - 1.8R	7.7	23.1	38.4	30.8	-	-
	pAM542 - 1.7P	2.8	27.8	36.1	33.3	-	-
	pAM542 - 1.7R	2.8	25.4	42.3	29.6	-	-
	<b>Synechococcus Transformants</b>						
25	pAM854	27.8	72.2	-	-	-	-
	pAM854 - $\Delta^{12}$	4.0	43.2	46.0	-	-	-
	pAM854 - $\Delta^6$	18.2	81.8	-	-	-	-
	pAM854 - $\Delta^6\&\Delta^{12}$	42.7	25.3	19.5	-	16.5	-
30	18:0, stearic acid; 18:1, oleic acid; 18:2, linoleic acid; 18:3( $\alpha$ ), linolenic acid; 18:3( $\gamma$ ), $\gamma$ -linolenic acid; 18:4, octadecatetraenoic acid						

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## EXAMPLE 4

Transformation of Synechococcus  
with  $\Delta 6$  and  $\Delta 12$  Desaturase Genes

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A third cosmid, cSy7, which contains a  $\Delta 12$ -desaturase gene, was isolated by screening the Synechocystis genomic library with a oligonucleotide synthesized from the published Synechocystis  $\Delta 12$ -desaturase gene sequence (Wada et al. [1990] Nature (London) 347, 200-203). A 1.7 kb AvaI fragment from this cosmid containing the  $\Delta 12$ -desaturase gene was identified and used as a probe to demonstrate that cSy13 not only contains a  $\Delta 6$ -desaturase gene but also a  $\Delta 12$ -desaturase gene (Figure 3). Genomic Southern blot analysis further showed that both the  $\Delta 6$ -and  $\Delta 12$ -desaturase genes are unique in the Synechocystis genome so that both functional genes involved in C18 fatty acid desaturation are linked closely in the Synechocystis genome.

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The unicellular cyanobacterium Synechococcus (PCC 7942) is deficient in both linoleic acid and GLA(3). The  $\Delta 12$  and  $\Delta 6$ -desaturase genes were cloned individually and together into pAM854 (Bustos et al. [1991] J. Bacteriol. 174, 7525-7533), a shuttle vector that contains sequences necessary for the integration of foreign DNA into the genome of Synechococcus (Golden et al. [1987] Methods in Enzymol. 153, 215-231). Synechococcus was transformed with these gene constructs and colonies were selected. Fatty acid methyl esters were extracted from transgenic Synechococcus and analyzed by GLC.

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1           Table 2 shows that the principal fatty acids  
of wild type Synechococcus are stearic acid (18:0) and  
oleic acid (18:1). Synechococcus transformed with  
pAM854- $\Delta$ 12 expressed linoleic acid (18:2) in addition  
5 to the principal fatty acids. Transformants with  
pAM854- $\Delta$ 6 and  $\Delta$ 12 produced both linoleate and GLA  
(Table 1). These results indicated that Synechococcus  
containing both  $\Delta$ 12- and  $\Delta$ 6-desaturase genes has  
gained the capability of introducing a second double  
10 bond at the  $\Delta$ 12 position and a third double bond at  
the  $\Delta$ 6 position of C18 fatty acids. However, no  
changes in fatty acid composition was observed in the  
transformant containing pAM854- $\Delta$ 6, indicating that in  
the absence of substrate synthesized by the  $\Delta$ 12  
15 desaturase, the  $\Delta$ 6-desaturase is inactive. This  
experiment further confirms that the 1.8 kb  
NheI/HindIII fragment (Figure 3) contains both coding  
and promoter regions of the Synechocystis  $\Delta$ 6-  
desaturase gene. Transgenic Synechococcus with  
20 altered levels of polyunsaturated fatty acids were  
similar to wild type in growth rate and morphology.

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## EXAMPLE 5

Nucleotide Sequence of  $\Delta 6$ -Desaturase

1 The nucleotide sequence of the 1.8 kb  
5 fragment of cSy75-3.5 including the functional  $\Delta 6$ -  
desaturase gene was determined. An open reading frame  
encoding a polypeptide of 359 amino acids was  
identified (Figure 4). A Kyte-Doolittle hydropathy  
analysis (Kyte et al. [1982] J. Mol. Biol. 157, 105-  
10 132) identified two regions of hydrophobic amino acids  
that could represent transmembrane domains (Figure  
1A); furthermore, the hydropathic profile of the  $\Delta 6$ -  
desaturase is similar to that of the  $\Delta 12$ -desaturase  
gene (Figure 1B; Wada et al.) and  $\Delta 9$ -desaturases  
15 (Thiede et al. [1986] J. Biol. Chem. 261, 13230-  
13235). However, the sequence similarity between the  
Synechocystis  $\Delta 6$ - and  $\Delta 12$ -desaturases is less than 40%  
at the nucleotide level and approximately 18% at the  
amino acid level. •

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## EXAMPLE 6

Transfer of Cyanobacterial  $\Delta^6$ -Desaturase into Tobacco

The cyanobacterial  $\Delta^6$ -desaturase gene was mobilized into a plant expression vector and transferred to tobacco using Agrobacterium mediated gene transfer techniques. To ensure that the transferred desaturase is appropriately expressed in leaves and developing seeds and that the desaturase gene product is targeted to the endoplasmic reticulum or the chloroplast, various expression cassettes with Synechocystis  $\Delta$ -desaturase open reading frame (ORF) were constructed. Components of these cassettes include: (i) a 35S promoter or seed specific promoter derived from the sunflower helianthinin gene to drive  $\Delta^6$ -desaturase gene expression in all plant tissues or only in developing seeds respectively, (ii) a putative signal peptide either from carrot extensin gene or sunflower helianthinin gene to target newly synthesized  $\Delta^6$ -desaturase into the ER, (iii) an ER lumen retention signal sequence (KDEL) at the COOH-terminal of the  $\Delta^6$ -desaturase ORF, and (iv) an optimized transit peptide to target  $\Delta^6$  desaturase into the chloroplast. The 35S promoter is a derivative of pRTL2 described by Restrepo et al. (1990). The optimized transit peptide sequence is described by Van de Broeck et al. (1985). The carrot extensin signal peptide is described by Chen et al (1985) EMBO J. 9, 2145.

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Transgenic tobacco plants were produced containing a chimeric cyanobacterial desaturase gene,

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1 comprised of the Synechocystis  $\Delta^6$  desaturase gene fused  
to an endoplasmic reticulum retention sequence (KDEL)  
and extensin signal peptide driven by the CaMV 35S  
promoter. PCR amplifications of transgenic tobacco  
5 genomic DNA indicate that the  $\Delta^6$  desaturase gene was  
incorporated into the tobacco genome. Fatty acid  
methyl esters of leaves of these transgenic tobacco  
plants were extracted and analyzed by Gas Liquid  
Chromatography (GLC). These transgenic tobacco  
10 accumulated significant amounts of GLA (Figure 4).  
Figure 4 shows fatty acid methyl esters as determined  
by GLC. Peaks were identified by comparing the  
elution times with known standards of fatty acid  
methyl ester. Accordingly, cyanobacterial genes  
15 involved in fatty acid metabolism can be used to  
generate transgenic plants with altered fatty acid  
compositions.

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## EXAMPLE 7

## Construction of Borage cDNA library

Membrane bound polysomes were isolated from  
5 borage seeds 12 days post pollination (12 DPP) using  
the protocol established for peas by Larkins and  
Davies (1975 Plant Phys. 55:749-756). RNA was  
extracted from the polysomes as described by Mechler  
(1987 Methods in Enzymology 152:241-248, Academic  
10 Press).

Poly-A+ RNA was isolated from the membrane  
bound polysomal RNA by use of Oligotex-dT beads  
(Qiagen). Corresponding cDNA was made using  
Stratagene's ZAP cDNA synthesis kit. The cDNA library  
15 was constructed in the lambda ZAP II vector  
(Stratagene) using the lambda ZAP II vector kit. The  
primary library was packaged in Gigapack II Gold  
packaging extract (Stratagene). The library was used  
to generate expressed sequence tags (ESTs), and  
20 sequences corresponding to the tags were used to scan  
the GenBank database.

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## EXAMPLE 8

## Hybridization Protocol

Hybridization probes for screening the  
5 borage cDNA library were generated by using random  
primed DNA synthesis as described by Ausubel et al  
(1994 Current Protocols in Molecular Biology, Wiley  
Interscience, N.Y.) and corresponded to previously  
identified abundantly expressed seed storage protein  
10 cDNAs. Unincorporated nucleotides were removed by use  
of a G-50 spin column (Boehringer Mannheim). Probe was  
denatured for hybridization by boiling in a water bath  
for 5 minutes, then quickly cooled on ice. Filters  
for hybridization were prehybridized at 60°C for 2-4  
15 hours in prehybridization solution (6XSSC [Maniatis et  
al 1984 Molecular Cloning A Laboratory Manual, Cold  
Spring Harbor Laboratory], 1X Denharts Solution, 0.05%  
sodium pyrophosphate, 100 µg/ml denatured salmon sperm  
DNA). Denatured probe was added to the hybridization  
20 solution (6X SSC, 1X Denharts solution, 0.05% sodium  
pyrophosphate, 100 µg/ml denatured salmon sperm DNA)  
and incubated at 60°C with agitation overnight.  
Filters were washed in 4x, 2x, and 1x SET washes for  
15 minutes each at 60°C. A 20X SET stock solution is  
25 3M NaCl, 0.4 M Tris base, 20 mM Na<sub>2</sub>EDTA-2H<sub>2</sub>O. The 4X  
SET wash was 4X SET, 12.5 mM PO<sub>4</sub>, pH 6.8 and 0.2% SDS.  
The 2X SET wash was 2X SET, 12.5 mM PO<sub>4</sub>, pH 6.8 and  
0.2% SDS. The 1X SET wash was 1X SET, 12.5 mM PO<sub>4</sub>, pH  
6.8 and 0.2% SDS. Filters were allowed to air dry and  
30 were then exposed to X-ray film for 24 hours with  
intensifying screens at -80°C.

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## EXAMPLE 9

Random sequencing of cDNAs from a borage seed  
(12 DPP) membrane-bound polysomal library

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The borage cDNA library was plated at low density (500 pfu on 150 mm petri dishes). Highly prevalent seed storage protein cDNAs were "subtracted" by screening with the previously identified corresponding cDNAs. Non-hybridizing plaques were excised using Stratagene's excision protocol and reagents. Resulting bacterial colonies were used to inoculate liquid cultures and were either sequenced manually or by an ABI automated sequencer. Each cDNA was sequenced once and a sequence tag generated from 200-300 base pairs. All sequencing was performed by cycle sequencing (Epicentre). Over 300 ESTs were generated. Each sequence tag was compared to GenBank database by BLASTX computer program and a number of lipid metabolism genes, including the  $\Delta 6$ -desaturase were identified.

Database searches with a cDNA clone designated mbp-65 using BLASTX with the GenBank database resulted in a significant match to the Synechocystis  $\Delta 6$ -desaturase. It was determined however, that this clone was not a full length cDNA. A full length cDNA was isolated using mbp-65 to screen the borage membrane-bound polysomal library. The sequence of the isolated cDNA was determined (Fig. 5A, SEQ ID NO:4) and the protein sequence of the open reading frame (Fig. 5B, SEQ ID NO:5) was compared to other known desaturases using Geneworks

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1 (IntelligGenetics) protein alignment program (Fig. 2).  
This alignment indicated that the cDNA was the borage  
Δ6-desaturase gene.

Although similar to other known plant  
5 desaturases, the borage delta 6-desaturase is distinct  
as indicated in the dendrogram shown in Fig. 6.  
Furthermore, comparison of the amino acid sequences  
characteristic of desaturases, particularly those  
proposed to be involved in metal binding (metal box 1  
10 and metal box 2), illustrates the differences between  
the borage delta 6-desaturase and other plant  
desaturases (Table 3).

The borage delta 6-desaturase is  
distinguished from the cyanobacterial form not only in  
15 over all sequence (Fig. 6) but also in the lipid box,  
metal box 1 and metal box 2 amino acid motifs (Table  
3). As Table 3 indicates, all three motifs are novel  
in sequence. Only the borage delta 6-desaturase metal  
box 2 shown some relationship to the Synechocystis  
20 delta-6 desaturase metal box 2.

In addition, the borage delta 6-desaturase  
is also distinct from another borage desaturase gene,  
the delta-12 desaturase. P1-81 is a full length cDNA  
that was identified by EST analysis and shows high  
25 similarity to the Arabidopsis delta-12 desaturase (Fad  
2). A comparison of the lipid box, metal box 1 and  
metal box 2 amino acid motifs (Table 3) in borage  
delta 6 and delta-12 desaturases indicates that little  
homology exists in these regions. The placement of  
30 the two sequences in the dendrogram in Fig. 6  
indicates how distantly related these two genes are.

Table 3. Comparison of common amino acid motifs in membrane-bound desaturases

Desaturase	Amino Acid Motif				Metal Box 1		Metal Box 2	
	Lipid Box	SEQ. ID. NO:	SEQ. ID. NO:	SEQ. ID. NO:	SEQ. ID. NO:	SEQ. ID. NO:	SEQ. ID. NO:	SEQ. ID. NO:
Borage $\Delta^6$	WIGHDAGH	(SEQ. ID. NO: 6)	HNAHH	(SEQ. ID. NO: 12)	FQIEHH	(SEQ. ID. NO: 20)		
Synechocystis $\Delta^6$	NVGHDANH	(SEQ. ID. NO: 7)	INYLHH	(SEQ. ID. NO: 13)	HQVTHH	(SEQ. ID. NO: 21)		
Arab. chloroplast $\Delta^{11}$	VLGHDCGH	(SEQ. ID. NO: 8)	HRTHH	(SEQ. ID. NO: 14)	HVIHH	(SEQ. ID. NO: 22)		
Rice $\Delta^{13}$	VLGHDCGH	(SEQ. ID. NO: 8)	HRTHH	(SEQ. ID. NO: 14)	HVIHH	(SEQ. ID. NO: 22)		
Glycine chloroplast $\Delta^{13}$	VLGHDCGH	(SEQ. ID. NO: 8)	HRTHH	(SEQ. ID. NO: 14)	HVIHH	(SEQ. ID. NO: 22)		
Arab. fad3 ( $\Delta^{13}$ )	VLGHDCGH	(SEQ. ID. NO: 8)	HRTHH	(SEQ. ID. NO: 14)	HVIHH	(SEQ. ID. NO: 22)		
Brassica fad3 ( $\Delta^{13}$ )	VLGHDCGH	(SEQ. ID. NO: 8)	HRTHH	(SEQ. ID. NO: 14)	HVIHH	(SEQ. ID. NO: 22)		
Borage $\Delta^{12}$ (PI-81)*	VIAHECGH	(SEQ. ID. NO: 9)	HRAHH	(SEQ. ID. NO: 15)	HVAHH	(SEQ. ID. NO: 23)		
Arab. fad2 ( $\Delta^{17}$ )	VIAHECGH	(SEQ. ID. NO: 9)	HRAHH	(SEQ. ID. NO: 15)	HVAHH	(SEQ. ID. NO: 23)		
Arab. chloroplast $\Delta^{11}$	VIGHDCAH	(SEQ. ID. NO: 10)	HDRHH	(SEQ. ID. NO: 16)	HIPHH	(SEQ. ID. NO: 24)		
Glycine plastid $\Delta^{12}$	VIGHDCAH	(SEQ. ID. NO: 10)	HDRHH	(SEQ. ID. NO: 16)	HIPHH	(SEQ. ID. NO: 24)		
Spinach plastidial n-6	VIGHDCAH	(SEQ. ID. NO: 10)	HDQRH	(SEQ. ID. NO: 17)	HIPHH	(SEQ. ID. NO: 24)		
Synechocystis $\Delta^{12}$	VVGHDCGH	(SEQ. ID. NO: 11)	IDHHH	(SEQ. ID. NO: 18)	HIPHH	(SEQ. ID. NO: 24)		
Anabaena $\Delta^{12}$	VLGHDCGH	(SEQ. ID. NO: 8)	HNHHH	(SEQ. ID. NO: 19)	HVPHH	(SEQ. ID. NO: 25)		

\*PI-81 is a full length cDNA which was identified by EST analysis and shows high similarity to the Arabidopsis  $\Delta 12$  desaturase (fad2)

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**EXAMPLE 10****Construction of 222.1Δ<sup>6</sup>NOS for transient  
and expression**

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The vector pBI221 (Jefferson et al. 1987  
EMBO J. 6:3901-3907) was prepared for ligation by  
digestion with BamHI and EcoICR I (Promega) which  
excises the GUS coding region leaving the 35S promoter  
and NOS terminator intact. The borage Δ 6-desaturase  
cDNA was excised from the Bluescript plasmid  
10 (Stratagene) by digestion with BamHI and XhoI. The  
XhoI end was made blunt by use of the Klenow fragment.  
This fragment was then cloned into the BamHI/EcoICR I  
sites of pBI221, yielding 221.Δ<sup>6</sup>NOS (Fig. 7). In  
221.Δ<sup>6</sup>.NOS, the remaining portion (backbone) of the  
15 restriction map depicted in Fig. 7 is pBI221.

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## EXAMPLE 11

Construction of 121. $\Delta^6$ .NOS for stable transformation

The vector pBI121 (Jefferson et al. 1987  
EMBO J. 6:3901-3907) was prepared for ligation by  
digestion with BamHI and EcoICR I (Promega) which  
excises the GUS coding region leaving the 35S promoter  
and NOS terminator intact. The borage  $\Delta^6$ -desaturase  
cDNA was excised from the Bluescript plasmid  
(Stratagene) by digestion with BamHI and XhoI. The  
XhoI end was made blunt by use of the Klenow fragment.  
This fragment was then cloned into the BamHI/EcoICR I  
sites of pBI121, yielding 121.1 $\Delta^6$ .NOS (Fig. 7). In  
121. $\Delta^6$ .NOS, the remaining portion (backbone) of the  
restriction map depicted in Fig. 7 is pBI121.

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## EXAMPLE 12

## Transient Expression

5 All work involving protoplasts was performed  
in a sterile hood. One ml of packed carrot suspension  
cells were digested in 30 mls plasmolyzing solution  
(25 g/l KCl, 3.5 g/l CaCl<sub>2</sub>·H<sub>2</sub>O, 10mM MES, pH 5.6 and  
0.2 M mannitol) with 1% cellulase, 0.1% pectolyase,  
and 0.1% dreisalase overnight, in the dark, at room  
10 temperature. Released protoplasts were filtered  
through a 150 µm mesh and pelleted by centrifugation  
(100x g, 5 min.) then washed twice in plasmolyzing  
solution. Protoplasts were counted using a double  
chambered hemocytometer. DNA was transfected into the  
15 protoplasts by PEG treatment as described by Nunberg  
and Thomas (1993 Methods in Plant Molecular Biology  
and Biotechnology, B.R. Glick and J.E. Thompson, eds.  
pp. 241-248) using 10<sup>6</sup> protoplasts and 50-70 ug of  
plasmid DNA (221.Δ6.NOS). Protoplasts were cultured  
20 in 5 mls of MS media supplemented with 0.2M mannitol  
and 3 µm 2,4-D for 48 hours in the dark with shaking.

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## EXAMPLE 13

## Stable transformation of tobacco

121.Δ<sup>6</sup>.NOS plasmid construction was used to transform tobacco (*Nicotiana tabacum* cv. xanthi) via Agrobacterium according to standard procedures (Horsh et al., 1985 Science 227: 1229-1231; Bogue et al., 1990 Mol. Gen. Genet. 221:49-57), except that initial transformants were selected on 100 ug/ml kanamycin.

## EXAMPLE 14

Preparation and analysis of  
fatty acid methyl esters (FAMES)

1 Tissue from transfected protoplasts and  
5 transformed tobacco plants was frozen in liquid  
nitrogen and lyophilized overnight. FAMES were  
prepared as described by Dahmer et al (1989 J. Amer.  
Oil Chem. Soc. 66:543-548). In some cases, the  
solvent was evaporated again, and the FAMES were  
10 resuspended in ethyl acetate and extracted once with  
deionized water to remove any water soluble  
contaminants. The FAMES were analyzed by gas  
chromatography (GC) on a J&W Scientific DB-wax column  
(30 m length, 0.25 mm ID, 0.25  $\mu$ m film).

15 An example of a transient assay is shown in  
Fig. 8 which represents three independent  
transfections pooled together. The addition of the  
borage  $\Delta 6$ -desaturase cDNA corresponds with the  
appearance of gamma linolenic acid (GLA) which is one  
20 of the possible products of  $\Delta 6$ -desaturase.

Figures 9 and 10 depict GC profiles of the  
FAMES derived from leaf and seed tissue, respectively,  
of control and transformed tobacco plants. Figure 9A  
provides the profile of leaf tissue of wild-type  
25 tobacco (xanthi); Figure 9B provides the profile of  
leaf tissue from a tobacco plant transformed with the  
borage  $\Delta$ -6 desaturase under the transcriptional  
control of the 35S CaMV promoter (pBI 121 $\Delta$ 'NOS).  
Peaks correspond to 18:2, 18:3 $\gamma$  (GLA), 18:3 $\alpha$  and 18:4  
30 (octadecanonic acid). Figure 10A shows the GC profile  
of seeds of a wild-type tobacco; Figure 10B shows the



- 1 profile of seed tissue of a tobacco plant transformed  
with pBI 121Δ<sup>6</sup>NOS. Peaks correspond to 18:2,  
18:3 $\gamma$ (GLA) and 18:3 $\alpha$ .

- 5 The relative distribution of the C<sub>18</sub> fatty  
acids in control and transgenic tobacco seeds is shown  
in Table 4.

TABLE 4

	Fatty Acid	Xanthi	pBI121Δ <sup>6</sup> NOS
10	18:0	4.0%	2.5%
	18:1	13%	13%
	18:2	82%	82%
	18:3 $\gamma$ (GLA)	-	2.7%
15	18:3 $\alpha$	0.82%	1.4%

- The foregoing results demonstrate that GLA  
is incorporated into the triacylglycerides of  
transgenic tobacco leaves and seeds containing the  
20 borage Δ<sup>6</sup>-desaturase.

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-44-

## SEQUENCE LISTING

## (1) GENERAL INFORMATION:

- (i) APPLICANT: Rhone-Poulenc Agrochimie
- (ii) TITLE OF INVENTION: PRODUCTION OF GAMMA LINOLENIC ACID BY A  
DELTA 6-DESATURASE
- (iii) NUMBER OF SEQUENCES: 25
- (iv) CORRESPONDENCE ADDRESS:
  - (A) ADDRESSEE: Scully, Scott, Murphy & Presser
  - (B) STREET: 400 Garden City Plaza
  - (C) CITY: Garden City
  - (D) STATE: New York
  - (E) COUNTRY: United States
  - (F) ZIP: 11530
- (v) COMPUTER READABLE FORM:
  - (A) MEDIUM TYPE: Floppy disk
  - (B) COMPUTER: IBM PC compatible
  - (C) OPERATING SYSTEM: PC-DOS/MS-DOS
  - (D) SOFTWARE: PatentIn Release #1.0, Version #1.25
- (vi) CURRENT APPLICATION DATA:
  - (A) APPLICATION NUMBER:
  - (B) FILING DATE: 30-DEC-1994
  - (C) CLASSIFICATION:
- (viii) ATTORNEY/AGENT INFORMATION:
  - (A) NAME: Presser, Leopold
  - (B) REGISTRATION NUMBER: 19,827
  - (C) REFERENCE/DOCKET NUMBER: 8383ZYXW
- (ix) TELECOMMUNICATION INFORMATION:
  - (A) TELEPHONE: (516) 742-4343
  - (B) TELEFAX: (516) 742-4366
  - (C) TELEX: 230 901 SANS UR

## (2) INFORMATION FOR SEQ ID NO:1:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 3588 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: both
  - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: DNA (genomic)

-45-

## (ix) FEATURE:

(A) NAME/KEY: CDS  
 (B) LOCATION: 2002..3081

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

GCTAGCCACC AGTGACGATG CTTGAATTT GGCCATTCTG ACCCAGGCC	GTATTCTGAA	60
TCCCCGCATT CGCATTGTTA ATCGTTTGTT CAACCATGCC CTGGGTAAAC	GTTTAGACAC	120
CACCTTGCCA GACCACGTTA GTTTGAGTGT TTCGGCCCTG GCGGCCCGA	TTTTTCCTT	180
TGCGGCTTTG GGCAATCAGG CGATCGGGCA ATTGCGTTTG TTTGACCAGA	CTTGGCCCAT	240
TCAGGAAATT GTCATTACCC AAGACCATCC CTGGCTCAAT TTACCCCTGG	CGGATTTATG	300
GGATGATCCG AGCCGAATGT TGATCTATTA CCTACCGGCC CACAGTGAAA	CGGATTTAGT	360
AGGCGCAGTG GTGAATAATT TAACGTTGCA ATCTGGGGAC CATTTAATAG	TGGGACAAA	420
ACCCCAACCC AAGACCAAAC GCGATCGCC TTGGCGCAA TTTTCAAAC	TGATTACCAA	480
CCTGCGGGAG TATCAGCGGT ATGTCCAACA GGTGATATGG GTGGTGTGT	TTTTATTGTT	540
GATGATTTTT CTGGCCACCT TCATCTACGT TTCCATTGAT CAACATATTG	CCCCAGTGGA	600
CGCGTTGTAT TTTTCGTGG GCATGATTAC CGGGCCCGT GGCAAGGAAG	AGGTGGCCGA	660
AAAGTCCCC GATATCATCA AAGTATTCAC AGTGTGATG ATGATCGCCG	GGGCGGGGT	720
GATTGGTATT TGTTATGCC TACTGAATGA TTTCATCCTT GGCAGTCGCT	TTAGTCAGTT	780
TTTGATGCG GCCAAGTTAC CCGATCGCCA TCACATCATC ATTTGTGGGC	TGGGGGAGT	840
GAGCATGGCC ATTATTGAAG AGTTAATTCA CCAGGGCCAT GAAATTGTGG	TAATCGAAAA	900
GGATACAGAT AATCGTTTCT TGCATACGGC CCGCTCCCTG GGGGTGCCCC	TAATTGTGGA	960
GGATGCCCCG CTAGAAAGAA CGTTGGCCTG CGCCAATATC AACCAGCCG	AAGCCATTGT	1020
GGTGCCACC AGCGACGACA CCGTTAACTT GGAAATTGGC CTAAGTCCA	AGGCGATCGC	1080
CCCTAGCCTG CCAGTGGTGT TCGTTGCCA GGATGCCCAG TTTAGCCTGT	CCCTGCAGGA	1140
AGTATTTGAA TTTGAAACGG TGCTTTGTCC GGCGGAATTG GCCACCTATT	CCTTTGCGGC	1200
GCGGCCCTG GGGGGCAAAA TTTTGGGCAA CGGCATGACC GATGATTGTC	TGTGGGTAGC	1260
CCTAGCCACC TTAATCACTC CTAACCATCC CTTTGCCGAC CAATTGGTTA	AAATTGCAGC	1320
CCAAAGTCT GATTTCGTTT CCTCTATCT AGAACGGGGT GGCAAAACCA	TCCATAGCTG	1380
GGAATTATTG GGTACCCATC TCGACTCTGG AGACGTGTTG TATTTAACCA	TGCCCCCAC	1440
TGCCCTAGAG CAACTTTGGC GATCGCCCCG TGCCACTGCT GATCCTCTGG	ACTCTTTTTT	1500

-46-

GGTTTAGCAT GGGGGGATGG AACTCTTGAC TCGGCCCAAT GGTGATCAAG AAAGAACGCT	1560
TTGTCTATGT TTAGTATTTT TAAGTTAACC AACAGCAGAG GATAACTTCC AAAAGAAATT	1620
AAGCTCAAAA AGTAGCAAAA TAAGTTTAAT TCATAACTGA GTTTTACTGC TAAACAGCGG	1680
TGCAAAAAAG TCAGATAAAA TAAAAGCTTC ACTTCGGTTT TATATTGTGA CCATGGTTCC	1740
CAGGCATCTG CTCTAGGGAG TTTTCCGCT GCCTTTAGAG AGTATTTTCT CCAAGTCGGC	1800
TAACTCCCCC ATTTTtaggc AAAATCATAT ACAGACTATC CCAATATTGC CAGAGCTTTG	1860
ATGACTCACT GTAGAAGGCA GACTAAAATT CTAGCAATGG ACTCCAGTT GGAATAAATT	1920
TTTAGTCTCC CCCGGCGCTG GAGTTTTTTT GTAGTTAATG GCGGTATAAT GTGAAAGTTT	1980
TTTATCTATT TAAATTTATA A ATG CTA ACA GCG GAA AGA ATT AAA TTT ACC Met Leu Thr Ala Glu Arg Ile Lys Phe Thr 1 5 10	2031
CAG AAA CGG GGG TTT CGT CGG GTA CTA AAC CAA CGG GTG GAT GCC TAC Gln Lys Arg Gly Phe Arg Arg Val Leu Asn Gln Arg Val Asp Ala Tyr 15 20 25	2079
TTT GCC GAG CAT GGC CTG ACC CAA AGG GAT AAT CCC TCC ATG TAT CTG Phe Ala Glu His Gly Leu Thr Gln Arg Asp Asn Pro Ser Met Tyr Leu 30 35 40	2127
AAA ACC CTG ATT ATT GTG CTC TGG TTG TTT TCC GCT TGG GCC TTT GTG Lys Thr Leu Ile Ile Val Leu Trp Leu Phe Ser Ala Trp Ala Phe Val 45 50 55	2175
CTT TTT GCT CCA GTT ATT TTT CCG GTG CGC CTA CTG GGT TGT ATG GTT Leu Phe Ala Pro Val Ile Phe Pro Val Arg Leu Leu Gly Cys Met Val 60 65 70	2223
TTG GCG ATC GCC TTG GCG GCC TTT TCC TTC AAT GTC GGC CAC GAT GCC Leu Ala Ile Ala Leu Ala Ala Phe Ser Phe Asn Val Gly His Asp Ala 75 80 85 90	2271
AAC CAC AAT GCC TAT TCC TCC AAT CCC CAC ATC AAC CGG GTT CTG GGC Asn His Asn Ala Tyr Ser Ser Asn Pro His Ile Asn Arg Val Leu Gly 95 100 105	2319
ATG ACC TAC GAT TTT GTC GGG TTA TCT AGT TTT CTT TGG CGC TAT CGC Met Thr Tyr Asp Phe Val Gly Leu Ser Ser Phe Leu Trp Arg Tyr Arg 110 115 120	2367
CAC AAC TAT TTG CAC CAC ACC TAC ACC AAT ATT CTT GGC CAT GAC GTG His Asn Tyr Leu His His Thr Tyr Thr Asn Ile Leu Gly His Asp Val 125 130 135	2415
GAA ATC CAT GGA GAT GGC GCA GTA CGT ATG AGT CCT GAA CAA GAA CAT Glu Ile His Gly Asp Gly Ala Val Arg Met Ser Pro Glu Gln Glu His 140 145 150	2463

-47-

GTT GGT ATT TAT CGT TTC CAG CAA TTT TAT ATT TGG GGT TTA TAT CTT Val Gly Ile Tyr Arg Phe Gln Gln Phe Tyr Ile Trp Gly Leu Tyr Leu 155 160 165 170	2511
TTC ATT CCC TTT TAT TGG TTT CTC TAC GAT GTC TAC CTA GTG CTT AAT Phe Ile Pro Phe Tyr Trp Phe Leu Tyr Asp Val Tyr Leu Val Leu Asn 175 180 185	2559
AAA GGC AAA TAT CAC GAC CAT AAA ATT CCT CCT TTC CAG CCC CTA GAA Lys Gly Lys Tyr His Asp His Lys Ile Pro Pro Phe Gln Pro Leu Glu 190 195 200	2607
TTA GCT AGT TTG CTA GGG ATT AAG CTA TTA TGG CTC GGC TAC GTT TTC Leu Ala Ser Leu Leu Gly Ile Lys Leu Leu Trp Leu Gly Tyr Val Phe 205 210 215	2655
GGC TTA CCT CTG GCT CTG GGC TTT TCC ATT CCT GAA GTA TTA ATT GGT Gly Leu Pro Leu Ala Leu Gly Phe Ser Ile Pro Glu Val Leu Ile Gly 220 225 230	2703
GCT TCG GTA ACC TAT ATG ACC TAT GGC ATC GTG GTT TGC ACC ATC TTT Ala Ser Val Thr Tyr Met Thr Tyr Gly Ile Val Val Cys Thr Ile Phe 235 240 245 250	2751
ATG CTG GCC CAT GTG TTG GAA TCA ACT GAA TTT CTC ACC CCC GAT GGT Met Leu Ala His Val Leu Glu Ser Thr Glu Phe Leu Thr Pro Asp Gly 255 260 265	2799
GAA TCC GGT GCC ATT GAT GAC GAG TGG GCT ATT TGC CAA ATT CGT ACC Glu Ser Gly Ala Ile Asp Asp Glu Trp Ala Ile Cys Gln Ile Arg Thr 270 275 280	2847
ACG GCC AAT TTT GCC ACC AAT AAT CCC TTT TGG AAC TGG TTT TGT GGC Thr Ala Asn Phe Ala Thr Asn Asn Pro Phe Trp Asn Trp Phe Cys Gly 285 290 295	2895
GGT TTA AAT CAC CAA GTT ACC CAC CAT CTT TTC CCC AAT ATT TGT CAT Gly Leu Asn His Gln Val Thr His His Leu Phe Pro Asn Ile Cys His 300 305 310	2943
ATT CAC TAT CCC CAA TTG GAA AAT ATT ATT AAG GAT GTT TGC CAA GAG Ile His Tyr Pro Gln Leu Glu Asn Ile Ile Lys Asp Val Cys Gln Glu 315 320 325 330	2991
TTT GGT GTG GAA TAT AAA GTT TAT CCC ACC TTC AAA GCG GCG ATC GCC Phe Gly Val Glu Tyr Lys Val Tyr Pro Thr Phe Lys Ala Ala Ile Ala 335 340 345	3039
TCT AAC TAT CGC TGG CTA GAG GCC ATG GGC AAA GCA TCG TGACATTGCC Ser Asn Tyr Arg Trp Leu Glu Ala Met Gly Lys Ala Ser 350 355 360	3088
TTGGGATTGA AGCAAAATGG CAAAATCCCT CGTAAATCTA TGATCGAAGC CTTTCTGTTG	3148
CCCGCCGACC AAATCCCCGA TGCTGACCAA AGGTTGATGT TGGCATTGCT CCAAACCCAC	3208

TTTGAGGGGG TTCATTGGCC GCAGTTTCAA GCTGACCTAG GAGGCAAAGA TTGGGTGATT 3268  
 TTGCTCAAAT CCGCTGGGAT ATTGAAAGGC TTCACCACCT TTGGTTTCTA CCCTGCTCAA 3328  
 TGGGAAGGAC AAACCGTCAG AATTGTTTAT TCTGGTGACA CCATCACCGA CCCATCCATG 3388  
 TGGTCTAACC CAGCCCTGGC CAAGGCTTGG ACCAAGGCCA TGCAAATTCT CCACGAGGCT 3448  
 AGGCCAGAAA AATTATATTG GCTCCTGATT TCTCCGGCT ATCGCACCTA CCGATTTTGT 3508  
 AGCATTTTTG CCAAGGAATT CTATCCCCAC TATCTCCATC CCACTCCCCC GCCTGTACAA 3568  
 AATTTTATCC ATCAGCTAGC 3588

## (2) INFORMATION FOR SEQ ID NO:2:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 359 amino acids  
 (B) TYPE: amino acid  
 (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: protein

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Met Leu Thr Ala Glu Arg Ile Lys Phe Thr Gln Lys Arg Gly Phe Arg  
 1 5 10 15  
 Arg Val Leu Asn Gln Arg Val Asp Ala Tyr Phe Ala Glu His Gly Leu  
 20 25 30  
 Thr Gln Arg Asp Asn Pro Ser Met Tyr Leu Lys Thr Leu Ile Ile Val  
 35 40 45  
 Leu Trp Leu Phe Ser Ala Trp Ala Phe Val Leu Phe Ala Pro Val Ile  
 50 55 60  
 Phe Pro Val Arg Leu Leu Gly Cys Met Val Leu Ala Ile Ala Leu Ala  
 65 70 75 80  
 Ala Phe Ser Phe Asn Val Gly His Asp Ala Asn His Asn Ala Tyr Ser  
 85 90 95  
 Ser Asn Pro His Ile Asn Arg Val Leu Gly Met Thr Tyr Asp Phe Val  
 100 105 110  
 Gly Leu Ser Ser Phe Leu Trp Arg Tyr Arg His Asn Tyr Leu His His  
 115 120 125  
 Thr Tyr Thr Asn Ile Leu Gly His Asp Val Glu Ile His Gly Asp Gly  
 130 135 140  
 Ala Val Arg Met Ser Pro Glu Gln Glu His Val Gly Ile Tyr Arg Phe  
 145 150 155 160

-49-

Gln Gln Phe Tyr Ile Trp Gly Leu Tyr Leu Phe Ile Pro Phe Tyr Trp  
 165 170 175  
 Phe Leu Tyr Asp Val Tyr Leu Val Leu Asn Lys Gly Lys Tyr His Asp  
 180 185 190  
 His Lys Ile Pro Pro Phe Gln Pro Leu Glu Leu Ala Ser Leu Leu Gly  
 195 200 205  
 Ile Lys Leu Leu Trp Leu Gly Tyr Val Phe Gly Leu Pro Leu Ala Leu  
 210 215 220  
 Gly Phe Ser Ile Pro Glu Val Leu Ile Gly Ala Ser Val Thr Tyr Met  
 225 230 235 240  
 Thr Tyr Gly Ile Val Val Cys Thr Ile Phe Met Leu Ala His Val Leu  
 245 250 255  
 Glu Ser Thr Glu Phe Leu Thr Pro Asp Gly Glu Ser Gly Ala Ile Asp  
 260 265 270  
 Asp Glu Trp Ala Ile Cys Gln Ile Arg Thr Thr Ala Asn Phe Ala Thr  
 275 280 285  
 Asn Asn Pro Phe Trp Asn Trp Phe Cys Gly Gly Leu Asn His Gln Val  
 290 295 300  
 Thr His His Leu Phe Pro Asn Ile Cys His Ile His Tyr Pro Gln Leu  
 305 310 315 320  
 Glu Asn Ile Ile Lys Asp Val Cys Gln Glu Phe Gly Val Glu Tyr Lys  
 325 330 335  
 Val Tyr Pro Thr Phe Lys Ala Ala Ile Ala Ser Asn Tyr Arg Trp Leu  
 340 345 350  
 Glu Ala Met Gly Lys Ala Ser  
 355

## (2) INFORMATION FOR SEQ ID NO:3:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1884 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: both
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: DNA (genomic)

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

AGCTTCACTT CGGTTTTATA TTGTGACCAT GGTTCCCAGG CATCTGCTCT AGGGAGTTTT 60  
 TCCGCTGCCT TTAGAGAGTA TTTTCTCCAA GTCGGCTAAC TCCCCCATTT TTAGGCAAAA 120

TCATATACAG	ACTATCCCAA	TATTGCCAGA	GCTTTGATGA	CTCACTGTAG	AAGGCAGACT	180
AAAATTCTAG	CAATGGACTC	CCAGTTGGAA	TAAATTTTTA	GTCTCCCCCG	GCGCTGGAGT	240
TTTTTTGTAG	TTAATGGCGG	TATAATGTGA	AAGTTTTTTA	TCTATTIAAA	TTTATAAATG	300
CTAACAGCGG	AAAGRAATTAA	ATTTACCCAG	AAACGGGGGT	TTCGTGGGT	ACTAAACCAA	360
CGGGTGGATG	CCTACTTTGC	CGAGCATGGC	CTGACCCAAA	GGGATAATCC	CTCCATGTAT	420
CTGAAAACCC	TGATTATTGT	GCTCTGGTTG	TTTCCGCTT	GGGCCTTTGT	GCTTTTTTGT	480
CCAGTTATTT	TTCCGGTGCG	CCTACTGGGT	TGTATGGTTT	TGGCGATCGC	CTTGGCGGCC	540
TTTTCTTCA	ATGTCGGCCA	CGATGCCAAC	CACAATGCCT	ATTCCTCCAA	TCCCCACATC	600
AACCGGGTTC	TGGGCATGAC	CTACGATTTT	GTCCGGTTAT	CTAGTTTTCT	TTGGCGCTAT	660
CGCCACAAC	ATTTGCACCA	CACCTACACC	AATATTCTTG	GCCATGACGT	GGAAATCCAT	720
GGAGATGGCG	CAGTACGTAT	GAGTCCTGAA	CAAGAACATG	TTGGTATTTA	TCGTTTCCAG	780
CAATTTTATA	TTTGGGGTTT	ATATCTTTTC	ATTCCCTTTT	ATTGGTTTCT	CTACGATGTC	840
TACCTAGTGC	TTAATAAAGG	CAAATATCAC	GACCATAAAA	TTCTCTCTTT	CCAGCCCCTA	900
GAATTAGCTA	GTTTGCTAGG	GATTAAGCTA	TTATGGCTCG	GCTACGTTTT	CGGCTTACCT	960
CTGGCTCTGG	GCTTTTCCAT	TCCTGAAGTA	TTAATTGGTG	CTTCGGTAAC	CTATATGACC	1020
TATGGCATCG	TGGTTTGCAC	CATCTTTATG	CTGGCCCATG	TGTTGGAATC	AACTGAATTT	1080
CTCACCCCCG	ATGGTGAATC	CGGTGCCATT	GATGACGAGT	GGGCTATTTG	CCAAATTCGT	1140
ACCACGGCCA	ATTTTGCCAC	CAATAATCCC	TTTTGGAACT	GGTTTTGTGG	CGGTTTAAAT	1200
CACCAAGTTA	CCCACCATCT	TTTCCCCAAT	ATTTGTCTATA	TTCACTATCC	CCAATTGGAA	1260
AATATTATTA	AGGATGTTTG	CCAAGAGTTT	GGTGTGGAAT	ATAAAGTTTA	TCCCACCTTC	1320
AAAGCGGCGA	TCGCCTCTAA	CTATCGCTGG	CTAGAGGCCA	TGGGCAAAGC	ATCGTGACAT	1380
TGCCTTGGA	TTGAAGCAAA	ATGGCAAAAT	CCCTCGTAAA	TCTATGATCG	AAGCCTTTCT	1440
GTTGCCCGCC	GACCAAATCC	CCGATGCTGA	CCAAAGGTTG	ATGTTGGCAT	TGCTCCAAAC	1500
CCACTTTGAG	GGGGTTTATT	GGCCGAGTT	TCAAGCTGAC	CTAGGAGGCA	AAGATTGGGT	1560
GATTTTGCTC	AAATCCGCTG	GGATATTGAA	AGGCTTCACC	ACCTTTGGTT	TCTACCCTGC	1620
TCAATGGGAA	GGACAAACCG	TCAGAAATTGT	TTATTCTGGT	GACACCATCA	CCGACCCATC	1680
CATGTGGTCT	AACCCAGCCC	TGGCCAAGGC	TTGGACCAAG	GCCATGCAAA	TTCTCCACGA	1740
GGCTAGGCCA	GAAAAATTAT	ATTGGCTCCT	GATTTCTTCC	GGCTATCGCA	CCTACCGATT	1800



TTTGAGCATT TTTGCCAAGG AATTCTATCC CCACTATCTC CATCCCACTC CCCCCCCTGT 1860  
 ACAAAATTTT ATCCATCAGC TAGC 1884

## (2) INFORMATION FOR SEQ ID NO:4:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1685 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: both
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: DNA (genomic)

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

AATATCTGCC TACCCTCCCA AAGAGAGTAG TCATTTTTC TCAATGGCTG CTCAAATCAA 60  
 GAAATACATT ACCTCAGATG AACTCAAGAA CCACGATAAA CCCGGAGATC TATGGATCTC 120  
 GATTCAAGGG AAAGCCTATG ATGTTTCGGA TTGGGTGAAA GACCATCCAG GTGGCAGCTT 180  
 TCCCTTGAAG AGTCTTGCTG GTCAAGAGGT AACTGATGCA TTTGTTGCAT TCCATCCTGC 240  
 CTCTACATGG AAGAATCTTG ATAAGTTTTT CACTGGGTAT TATCTTAAAG ATTACTCTGT 300  
 TTCTGAGGTT TCTAAAGATT ATAGGAAGCT TGTGTTTGAG TTTTCTAAAA TGGGTTTGTA 360  
 TGACAAAAAA GGTCATATTA TGTTTGCAAC TTTGTGCTTT ATAGCAATGC TGTTTGCTAT 420  
 GAGTGTTTAT GGGGTTTTGT TTTGTGAGGG TGTTTTGTA CATTTGTTTT CTGGGTGTTT 480  
 GATGGGGTTT CTTTGGATTC AGAGTGGTTG GATTGGACAT GATGCTGGGC ATTATATGGT 540  
 AGTGTCTGAT TCAAGGCTTA ATAAGTTTAT GGGTATTTTT GCTGCAAATT GTCTTTCAGG 600  
 AATAAGTATT GGTGGGTGGA AATGGAACCA TAATGCACAT CACATTGCCT GTAATAGCCT 660  
 TGAATATGAC CCTGATTTAC AATATATACC ATTCCTTGTT GTGTCTTCCA AGTTTTTTGG 720  
 TCACTCACC TCTCATTTCT ATGAGAAAAG GTTGACTTTT GACTCTTTAT CAAGATTCTT 780  
 TGTAAAGTTAT CAACATTGGA CATTTTACCC TATTATGTGT GCTGCTAGGC TCAATATGTA 840  
 TGTACAATCT CTCATAATGT TGTGACCAA GAGAAATGTG TCCTATCGAG CTCAGGAACT 900  
 CTTGGGATGC CTAGTGTCT CGATTTGGTA CCCGTGCTT GTTCTTGT TGCCTAATTG 960  
 GGGTGAAAGA ATTATGTTTG TTATTGCAAG TTTATCAGTG ACTGGAATGC AACAAGTTCA 1020  
 GTTCTCCTTG AACCATTCT CTTCAAGTGT TTATGTTGGA AAGCCTAAAG GGAATAATTG 1080  
 GTTTGAGAAA CAAACGGATG GGACACTTGA CATTTCTTGT CCTCCTTGA TGGATTGTTT 1140  
 TCATGGTGA TTGCAATTCC AAATTGAGCA TCATTGTTT CCAAGATGC CTAGATGCAA 1200

```

CCTTAGGAAA ATCTCGCCCT ACGTGATCGA GTTATGCAAG AAACATAATT TGCCTTACAA      1260
TTATGCATCT TTCTCCAAGG CCAATGAAAT GACACTCAGA ACATTGAGGA ACACAGCATT      1320
GCAGGCTAGG GATATAACCA AGCCGCTCCC GAAGAATTG GTATGGGAAG CTCTTCACAC      1380
TCATGGTTAA AATTACCCCT AGTTCATGTA ATAATTTGAG ATTATGTATC TCCTATGTTT      1440
GTGTCTTGTC TTGGTTCTAC TTGTTGGAGT CATTGCAACT TGTCTTTTAT GGTTTATTAG      1500
ATGTTTTTTA ATATATTTTA GAGGTTTTGC TTTTCATCTCC ATTATGATG AATAAGGAGT      1560
TGCATATTGT CAATTGTGTG GCTCAATATC TGATATTTTG GAATGTACTT TGTACCACTG      1620
TGTTTTTCAGT TGAAGCTCAT GTGTACTTCT ATAGACTTTG TTTAAATGGT TATGTCATGT      1680
TATTT                                             1685

```

## (2) INFORMATION FOR SEQ ID NO:5:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 448 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: DNA (genomic)

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

```

Met Ala Ala Gln Ile Lys Lys Tyr Ile Thr Ser Asp Glu Leu Lys Asn
1           5           10           15
His Asp Lys Pro Gly Asp Leu Trp Ile Ser Ile Gln Gly Lys Ala Tyr
20          25          30
Asp Val Ser Asp Trp Val Lys Asp His Pro Gly Gly Ser Phe Pro Leu
35          40          45
Lys Ser Leu Ala Gly Gln Glu Val Thr Asp Ala Phe Val Ala Phe His
50          55          60
Pro Ala Ser Thr Trp Lys Asn Leu Asp Lys Phe Phe Thr Gly Tyr Tyr
65          70          75          80
Leu Lys Asp Tyr Ser Val Ser Glu Val Ser Lys Asp Tyr Arg Lys Leu
85          90          95
Val Phe Glu Phe Ser Lys Met Gly Leu Tyr Asp Lys Lys Gly His Ile
100         105         110
Met Phe Ala Thr Leu Cys Phe Ile Ala Met Leu Phe Ala Met Ser Val
115         120         125
Tyr Gly Val Leu Phe Cys Glu Gly Val Leu Val His Leu Phe Ser Gly
130         135         140

```

-53-

Cys Leu Met Gly Phe Leu Trp Ile Gln Ser Gly Trp Ile Gly His Asp  
 145 150 155 160  
 Ala Gly His Tyr Met Val Val Ser Asp Ser Arg Leu Asn Lys Phe Met  
 165 170 175  
 Gly Ile Phe Ala Ala Asn Cys Leu Ser Gly Ile Ser Ile Gly Trp Trp  
 180 185 190  
 Lys Trp Asn His Asn Ala His His Ile Ala Cys Asn Ser Leu Glu Tyr  
 195 200 205  
 Asp Pro Asp Leu Gln Tyr Ile Pro Phe Leu Val Val Ser Ser Lys Phe  
 210 215 220  
 Phe Gly Ser Leu Thr Ser His Phe Tyr Glu Lys Arg Leu Thr Phe Asp  
 225 230 235 240  
 Ser Leu Ser Arg Phe Phe Val Ser Tyr Gln His Trp Thr Phe Tyr Pro  
 245 250 255  
 Ile Met Cys Ala Ala Arg Leu Asn Met Tyr Val Gln Ser Leu Ile Met  
 260 265 270  
 Leu Leu Thr Lys Arg Asn Val Ser Tyr Arg Ala Gln Glu Leu Leu Gly  
 275 280 285  
 Cys Leu Val Phe Ser Ile Trp Tyr Pro Leu Leu Val Ser Cys Leu Pro  
 290 295 300  
 Asn Trp Gly Glu Arg Ile Met Phe Val Ile Ala Ser Leu Ser Val Thr  
 305 310 315 320  
 Gly Met Gln Gln Val Gln Phe Ser Leu Asn His Phe Ser Ser Ser Val  
 325 330 335  
 Tyr Val Gly Lys Pro Lys Gly Asn Asn Trp Phe Glu Lys Gln Thr Asp  
 340 345 350  
 Gly Thr Leu Asp Ile Ser Cys Pro Pro Trp Met Asp Trp Phe His Gly  
 355 360 365  
 Gly Ser Gln Phe Gln Ile Glu His His Leu Phe Pro Lys Met Pro Arg  
 370 375 380  
 Cys Asn Leu Arg Lys Ile Ser Pro Tyr Val Ile Glu Leu Cys Lys Lys  
 385 390 395 400  
 His Asn Leu Pro Tyr Asn Tyr Ala Ser Phe Ser Lys Ala Asn Glu Met  
 405 410 415  
 Thr Leu Arg Thr Leu Arg Asn Thr Ala Leu Gln Ala Arg Asp Ile Thr  
 420 425 430  
 Lys Pro Leu Pro Lys Asn Leu Val Trp Glu Ala Leu His Thr His Gly  
 435 440 445

## (2) INFORMATION FOR SEQ ID NO:6:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 8 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: DNA (genomic)

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

Trp Ile Gly His Asp Ala Gly His  
1 5

## (2) INFORMATION FOR SEQ ID NO:7:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 8 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: DNA (genomic)

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

Asn Val Gly His Asp Ala Asn His  
1 5

## (2) INFORMATION FOR SEQ ID NO:8:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 8 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: DNA (genomic)

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

Val Leu Gly His Asp Cys Gly His  
1 5

## (2) INFORMATION FOR SEQ ID NO:9:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 8 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: DNA (genomic)

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

-55-

Val Ile Ala His Glu Cys Gly His  
1 5

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 8 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

Val Ile Gly His Asp Cys Ala His  
1 5

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 8 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

Val Val Gly His Asp Cys Gly His  
1 5

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 5 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

His Asn Ala His His  
1 5

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 6 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

-56-

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

His Asn Tyr Leu His His  
1 5

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 5 amino acids  
(B) TYPE: amino acid  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

His Arg Thr His His  
1 5

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 5 amino acids  
(B) TYPE: amino acid  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

His Arg Arg His His  
1 5

-57-

## (2) INFORMATION FOR SEQ ID NO:16:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 5 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: DNA (genomic)

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

His Asp Arg His His  
1 5

## (2) INFORMATION FOR SEQ ID NO:17:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 5 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: DNA (genomic)

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

His Asp Gln His His  
1 5

## (2) INFORMATION FOR SEQ ID NO:18:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 5 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: DNA (genomic)

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

His Asp His His His  
1 5

## (2) INFORMATION FOR SEQ ID NO:19:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 5 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: DNA (genomic)

-58-

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

His Asn His His His  
1 5

(2) INFORMATION FOR SEQ ID NO:20:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 6 amino acids  
(B) TYPE: amino acid  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

Phe Gln Ile Glu His His  
1 5

(2) INFORMATION FOR SEQ ID NO:21:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 6 amino acids  
(B) TYPE: amino acid  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

His Gln Val Thr His His  
1 5

(2) INFORMATION FOR SEQ ID NO:22:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 5 amino acids  
(B) TYPE: amino acid  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

His Val Ile His His  
1 5

(2) INFORMATION FOR SEQ ID NO:23:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 5 amino acids  
(B) TYPE: amino acid



-59-

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

His Val Ala His His  
1 5

(2) INFORMATION FOR SEQ ID NO:24:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 5 amino acids  
(B) TYPE: amino acid  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:24:

His Ile Pro His His  
1 5

(2) INFORMATION FOR SEQ ID NO:25:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 5 amino acids  
(B) TYPE: amino acid  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:25:

His Val Pro His His  
1 5

1 WHAT IS CLAIMED:

1. An isolated nucleic acid encoding a borage  $\Delta 6$ -desaturase.
2. The isolated nucleic acid of Claim 1 comprising the nucleotide sequence of SEQ ID NO: 4.
3. An isolated nucleic acid that codes for the amino acid sequence of SEQ ID NO: 5.
4. A vector comprising the nucleic acid of any one Claims 1-3.
5. An expression vector comprising the isolated nucleic acid of any one of Claims 1-3 operably linked to a promoter and optionally a termination signal capable of effecting expression of the gene product of said isolated nucleic acid.
6. The expression vector of Claim 5 wherein said promoter is a  $\Delta$ -6 desaturase promoter, an Anabaena carboxylase promoter, a helianthinin promoter, a glycinin promoter, a napin promoter, the 35S promoter from CaMV, or a helianthinin tissue-specific promoter.
7. The expression vector of Claim 5 wherein said promoter is constitutive or tissue-specific.
8. The expression vector of Claim 5 wherein said termination signal is a Synechocystis termination

1 signal, a nopaline synthase termination signal, or a seed  
termination signal.

9. A cell comprising the vector of any one of  
5 Claims 4-8.

10. The cell of Claim 9 wherein said cell is an  
animal cell, a bacterial cell, a plant cell or a fungal  
cell.

10

11. A transgenic organism comprising the  
isolated nucleic acid of any one of Claims 1-3.

12. A transgenic organism comprising the vector  
15 of any one of Claims 4-8.

13. The transgenic organism of Claim 11 or 12  
wherein said organism is a bacterium, a fungus, a plant or  
an animal.

20

14. A plant or progeny of said plant which has  
been regenerated from the plant cell of Claim 10.

15. The plant of Claim 14 wherein said plant is  
25 a sunflower, soybean, maize, tobacco, peanut, carrot or  
oil seed rape plant.

16. A method of producing a plant with  
increased gamma linolenic acid (GLA) content which  
30 comprises:

35

1           (a) transforming a plant cell with the isolated  
nucleic acid of any one of Claims 1-3; and

          (b) regenerating a plant with increased GLA  
content from said plant cell.

5

17. A method of producing a plant with  
increased gamma linolenic acid (GLA) content which  
comprises:

          (a) transforming a plant cell with the vector of  
10 any one of Claims 4-8; and

          (b) regenerating a plant with increased GLA  
content from said plant cell.

18. The method of Claim 16 or 17 wherein said  
15 plant is a sunflower, soybean, maize, tobacco, peanut,  
carrot or oil seed rape plant.

19. A method of inducing production of gamma  
linolenic acid (GLA) in an organism deficient or lacking  
20 in GLA which comprises transforming said organism with the  
isolated nucleic acid of any one of Claims 1-3.

20. A method of inducing production of gamma  
linolenic acid (GLA) in an organism deficient or lacking  
25 in GLA which comprises transforming said organism with the  
vector of any one of Claims 4-8.

21. A method of inducing production of gamma  
linolenic acid (GLA) in an organism deficient or lacking  
30 in GLA and linoleic acid (LA) which comprises transforming  
said organism with an isolated nucleic acid encoding

35

- 1 borage  $\Delta 6$ -desaturase and an isolated nucleic acid encoding  
 $\Delta 12$ -desaturase.

22. The method of Claim 21 wherein said  
5 isolated nucleic acid encoding  $\Delta 6$ -desaturase comprises  
nucleotides 44 to 1390 of SEQ. ID NO: 4.

23. A method of inducing production of  
octadecatetraeonic acid in an organism deficient or  
10 lacking in gamma linolenic acid which comprises  
transforming said organism with the isolated nucleic acid  
of any one of Claims 1-3.

24. A method of inducing production of  
15 octadecatetraeonic acid in an organism deficient or  
lacking in gamma linolenic acid which comprises  
transforming said organism with the vector of any one of  
Claims 4-8.

- 20 25. The method of Claim 23 or 24 wherein said  
organism is a bacterium, a fungus, a plant or an animal.

26. A method of producing a plant with improved  
chilling resistance which comprises:

- 25 (a) transforming a plant cell with the isolated  
nucleic acid of any one of Claims 1-3; and  
(b) regenerating said plant with improved  
chilling resistance from said transformed plant cell.

- 30 27. A method of producing a plant with improved  
chilling resistance which comprises:

1 (a) transforming a plant cell with the vector of  
any one of Claims 4-8; and

(b) regenerating said plant with improved  
chilling resistance from said transformed plant cell.

5

28. The method of Claim 26 or 27 wherein said  
plant is a sunflower, soybean, maize, tobacco, peanut,  
carrot or oil seed rape plant.

10

15

20

25

30

35

1 / 9

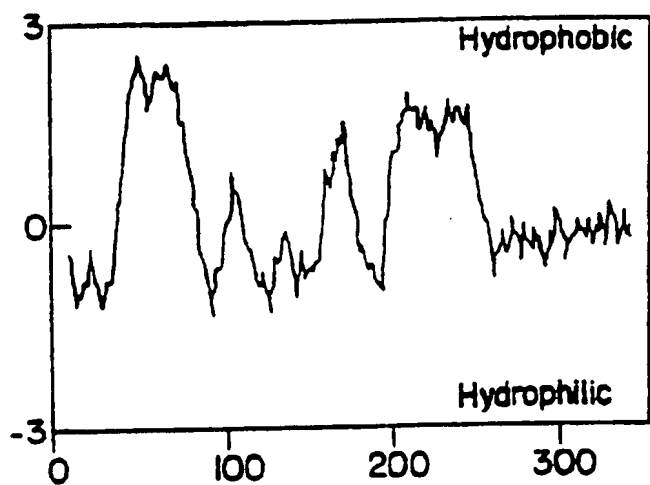


FIG. IA

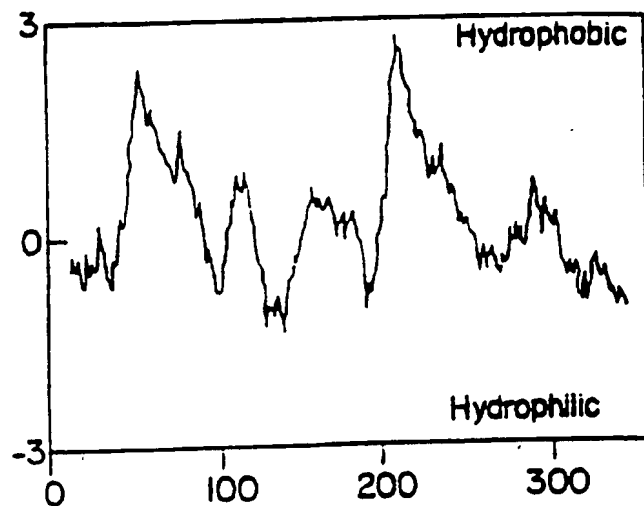
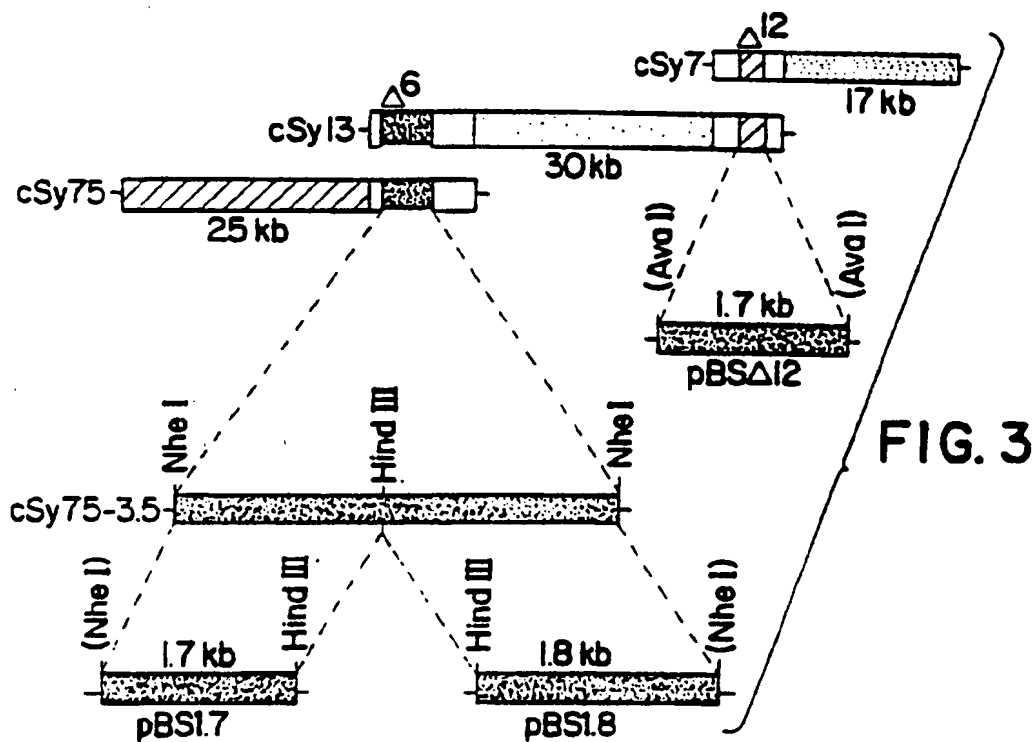
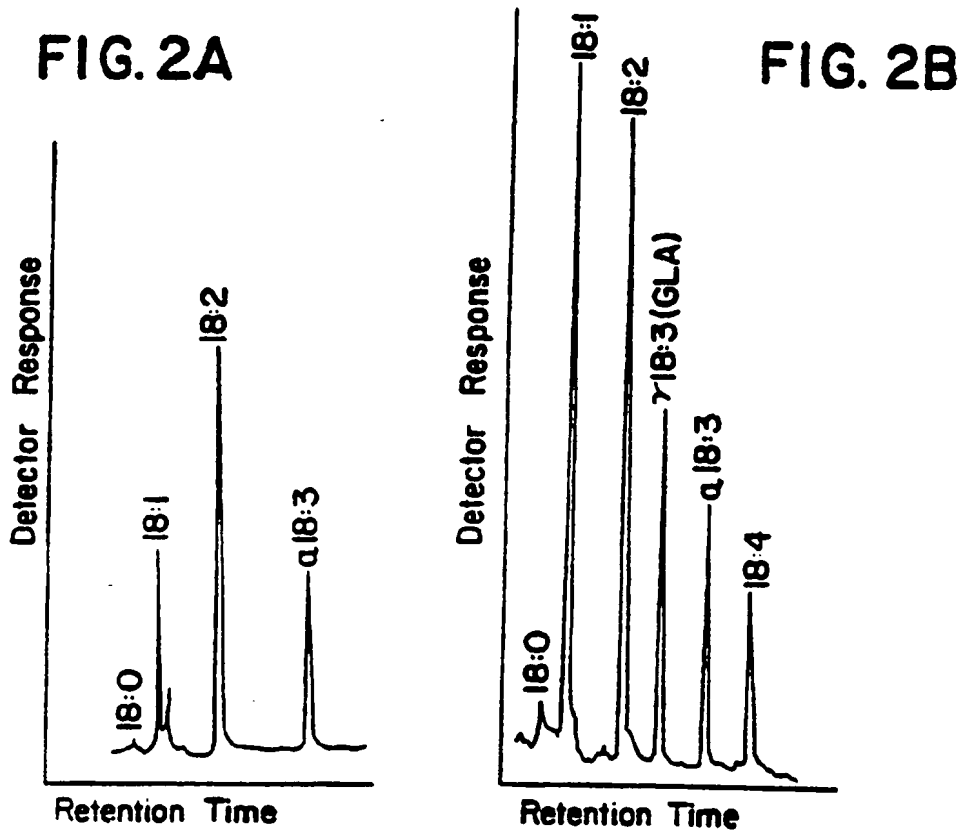


FIG. IB

2 / 9



SUBSTITUTE SHEET (RULE 26)



3 / 9

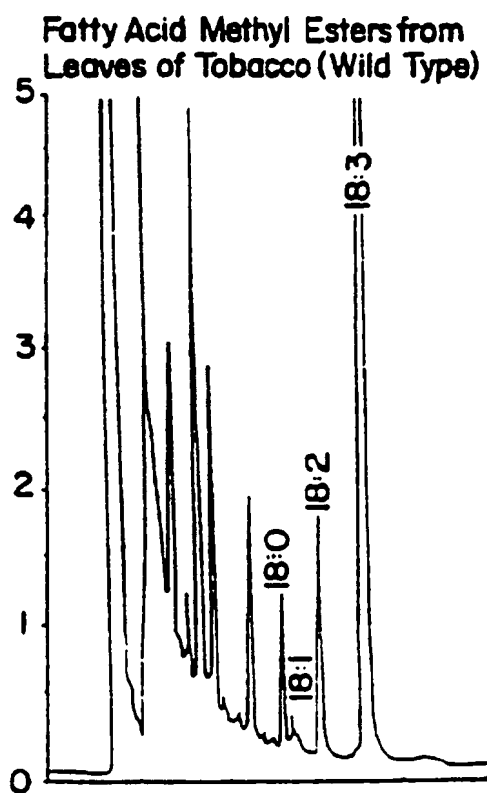


FIG. 4A

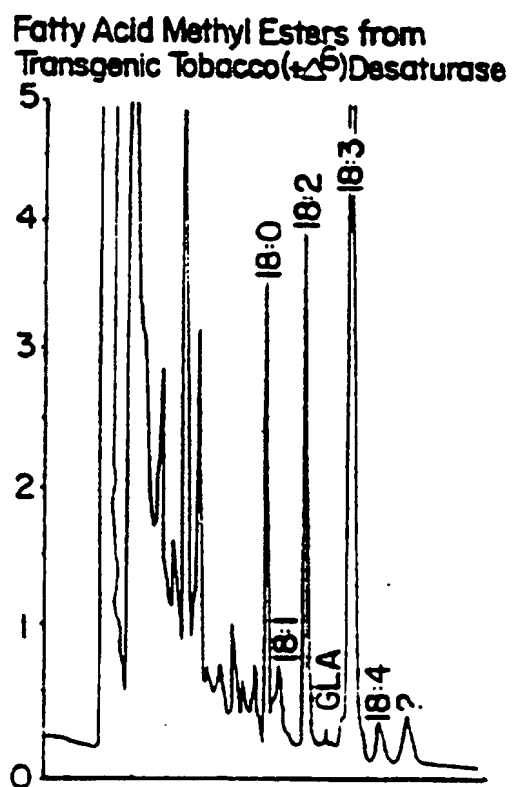


FIG. 4B

FIG.5A

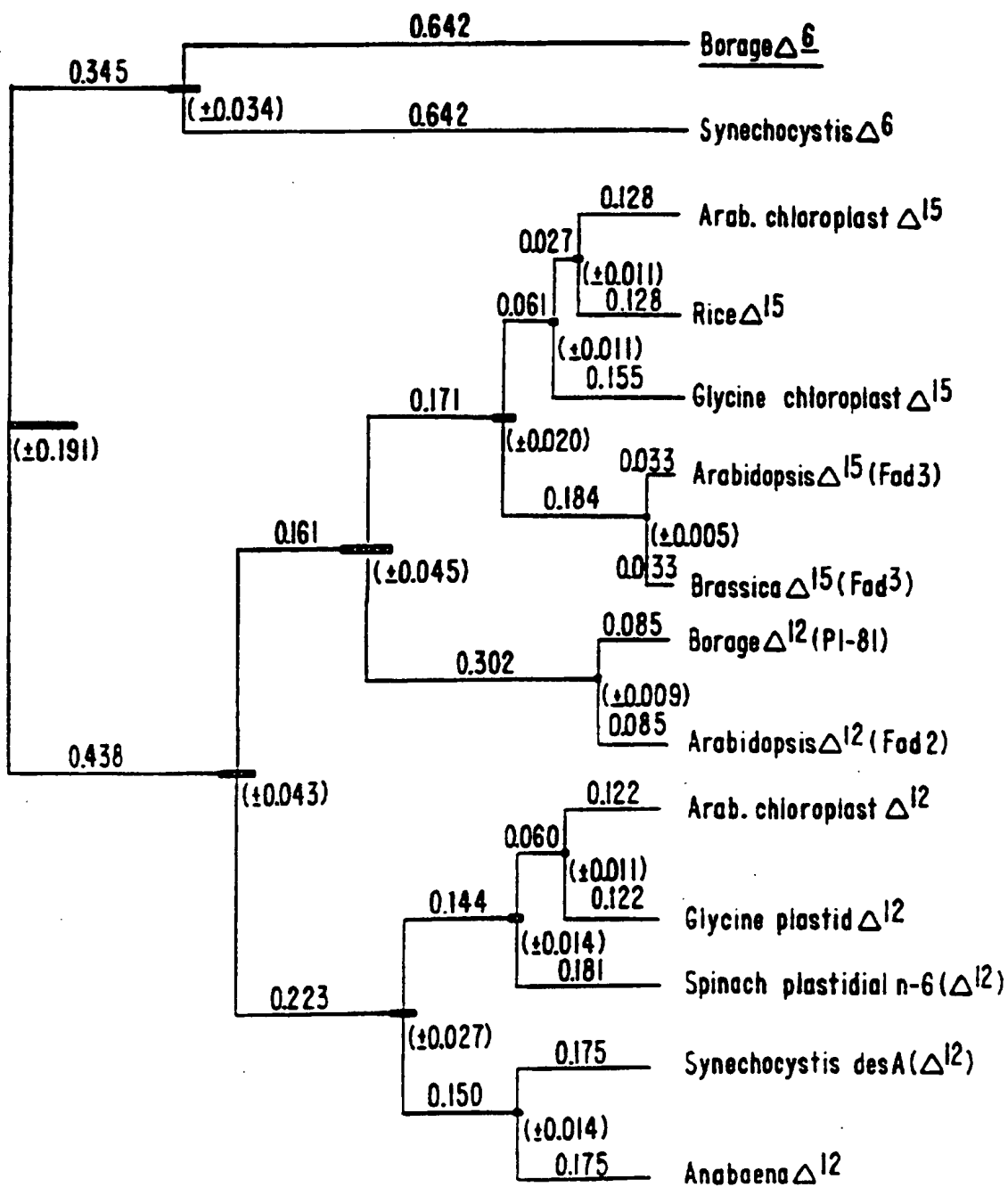
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 161 gaccatccag gtggcagctt tcccttgaag agtcttgctg  
 241 ctctacatg9 aagaatcttg ataagttttt cactgggtat  
 321 atagggaagct t9tgcttgag ttttctaaa tgggttttga  
 401 atagcaatgc t9tttgctat gagtgtttat g9ggttttgc  
 481 gatggggttt ctttggattc agagtgggtg gattggacat  
 561 ataaagttaa ggttattttt gctgcaaat gcttttcagg  
 641 cacattgcct gtaatagcct tgaatatgac cctgattttac  
 721 ttcactcacc tctcatttct atgagaaaa gttgactttt  
 801 cattttacc tattatgtgt gctgctaggc tcaatatgta  
 881 tcctatcgag ctcaggaaact cttgggagtc ctagtgttct  
 961 ggtgaaaga attatgtttg ttattgcaag ttatcagtg  
 1041 cttcaagtgt ttatgttggg aagcctaaa ggaataattg  
 1121 cctccttggg tggattgggt tcatggtgga ttgcaattcc  
 1201 ccttaggaaa atctcgccct acgtgatcga ttatgcaag  
 1281 ccaatgaaat gacactcaga acattgagga acacagcatt  
 1361 gtatgggaag ctcttcacac tcatggttaa aattaccctt  
 1441 gtgtcttgtc ttggttctac ttggtggagt cattgcaact  
 1521 gagggtttgc tttcatctcc attattgatg aataaggagt  
 1601 gaatgtactt t9taccactg t9ttttcagt t9aagctcat  
 1681 tattt  
 tcaatggctg ctcaaatcaa gaaatacatt acctcagatg 80  
 gattcaagg9 aaagcctatg atgtttc9ga ttgggtgaaa 160  
 gtcaagaggt aactgatgca ttgtttgcac tccatcctgc 240  
 tatcttaaag attactctgt ttctgagggt tctaagatc 320  
 tgacaaaaaa ggtcataatta t9tttgcaac ttgtgcttt 400  
 ttgtgagg9 t9ttttggta catttggttt ctgggtggtt 480  
 gatgtg9gc attatatggt agtgtctgat tcaaggcctta 560  
 aataagtatt g9ttg9tgga aatggaacca taatgcacat 640  
 aatatatacc attccttgtt g9tcttcca agttttttg 720  
 gactctttat caagattctt t9taagttat caacattgga 800  
 t9tacaatct ctcataatgt t9ttgaccaa gagaatgtg 880  
 cgatttggtg cccgttgctt gtttctgtt t9cctaattg 960  
 actggaatgc aacaagtcca gttctccttg aaccacttct 1040  
 gtttgagaaa caaacggatg ggacacttga catttcttgt 1120  
 aaattgagca tcatttgtt cccaagatgc ctatagcaaa 1200  
 aaacataat t9ccttaca ttatgcatct tcttccaagg 1280  
 gcaggctagg gataaacca agccgctccc gaagaatttg 1360  
 agttcatgta ataatttgag attatgtatc tcttatgttt 1440  
 t9tcttttat ggtttattag atgtttttta atatatttta 1520  
 t9cataattg caattgtgt gctcaatatc t9atatatttg 1600  
 g9tacttct atagactttg tttaaatggt tatgtcatgt 1680  
 1685

FIG.5B

1 MAAQIKKYIT SDELKNHDKP GDLWISIQGK AYDVSMDWKD HPGSFPPLKS LAGQEVTDAF VAFHPASTWK NLDKFFTGYY 80  
 81 LKDYSVSEVS KDYRKLVEEF SKMGLYDKKG HINFATLCFI AMLFAMSVYG VLFCEGVLVH LFSGCLMGFL WIQSGWIGHD 160  
 161 AGHYMVVSDS RLNKFMGIFA ANCLSGISIG WKKWNHNAHH IACNSLEYDP DLQYIPFLV SSKFFGSLTS HFYEKRLTFD 240  
 241 SLSRFFVSQY HWTYPIMCA ARLNMYVQSL IMLLTKRNV YRAQELLGCL VFSIWYPLL SCLPNWGERI MFVIASLSVT 320  
 321 GMQVQFSLN HFSSSVYVGK PKGNWFEKQ TDGTLDISCP PMMDWFHGLL QFOIEHHHLLFP KNPRCNLRKI SPYVIELCKK 400  
 401 HNLPPNYASF SKANEMTLRT LRNTALQARD ITKPLPKNLV WEALHTHG 448

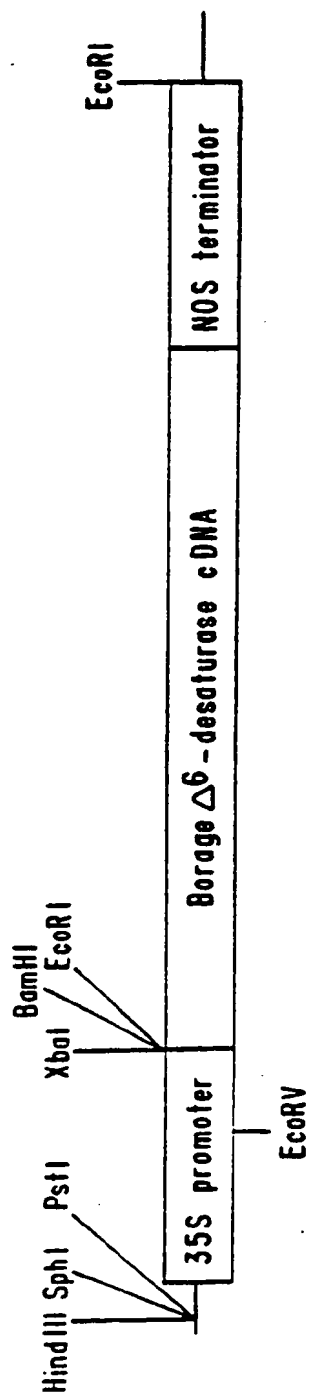
5 / 9

FIG. 6



6 / 9

FIG. 7



7 / 9

FIG. 8A

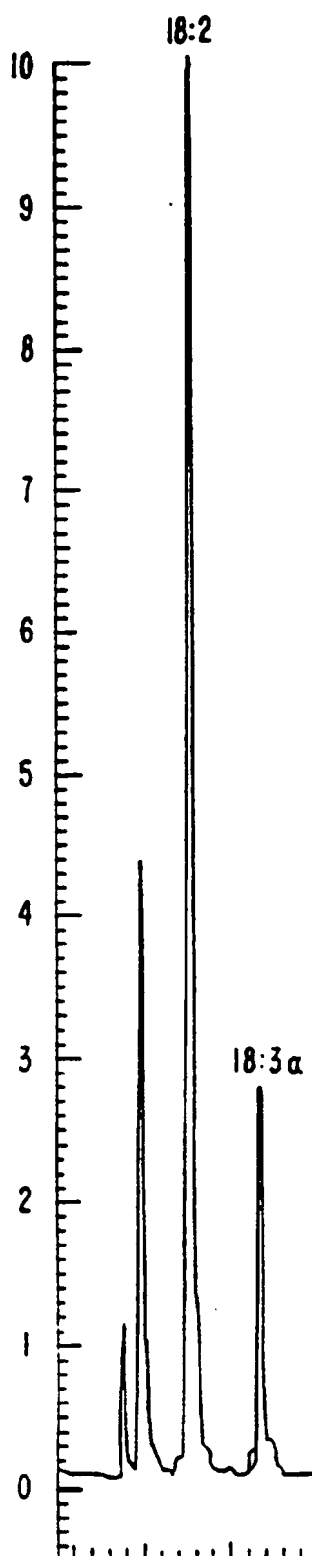


FIG. 8B

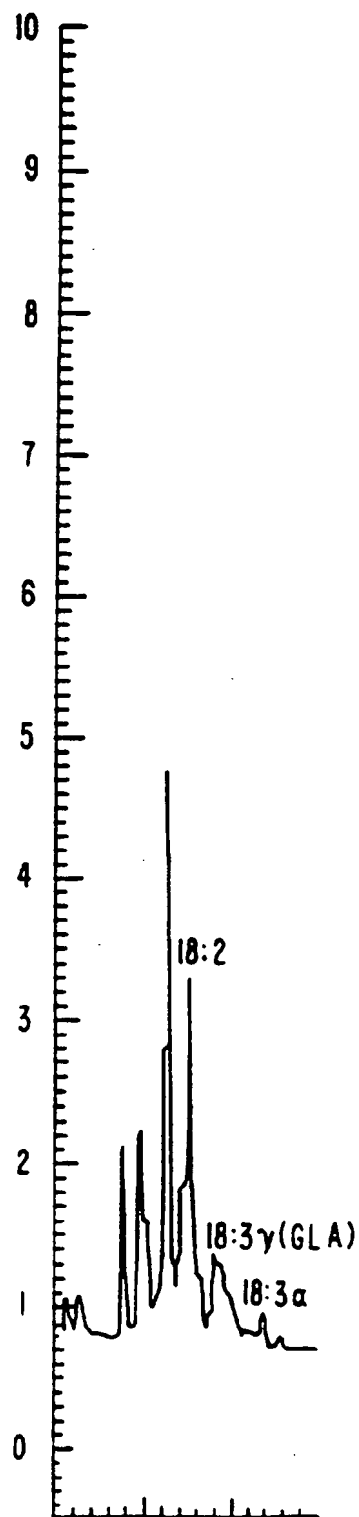
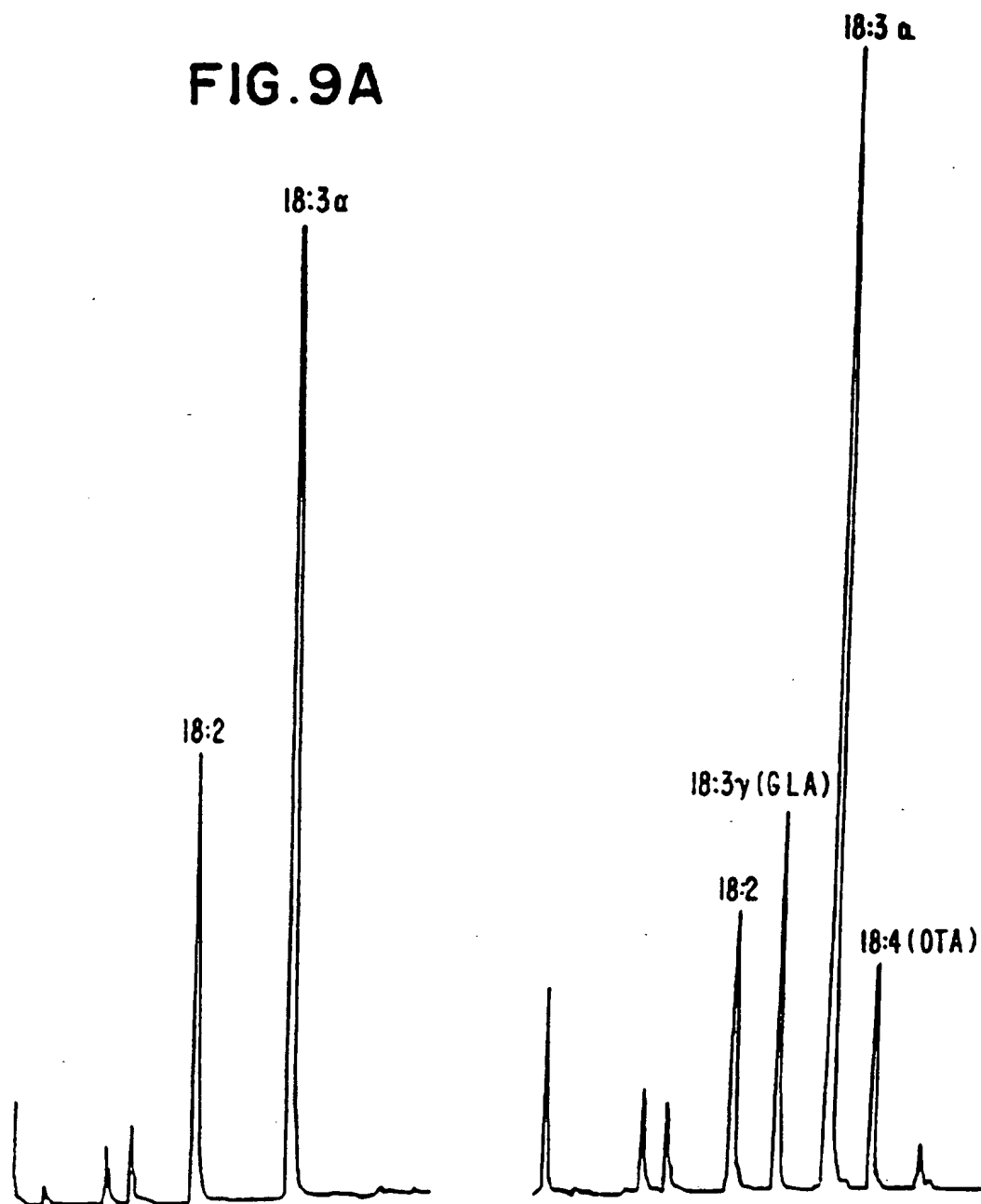


FIG. 9B

FIG. 9A



9 / 9

FIG. 10A

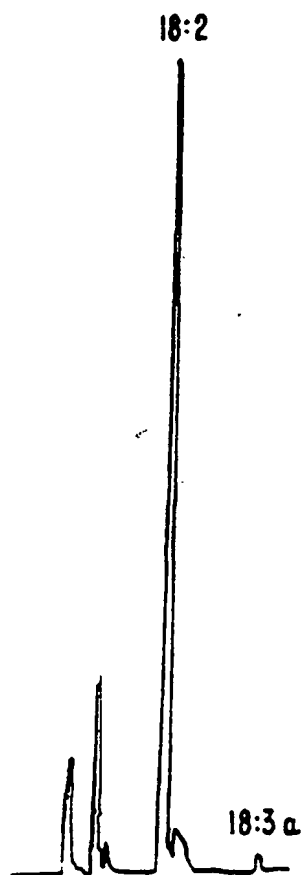


FIG. 10B

